MONITORING STRESS HORMONES IN REHABILITATED AND CAPTIVE OTARIIDS

By

Lisa Petrauskas

RECOMMENDED:

Advisory Committee Co-Chair

Advisory Committee Co-Chair

Head, Program in Marine Science and Limnology

APPROVED:

Dean, School of Fisheries and Ocean Sciences

Dean of the Graduate School

8/4/05

Date

MONITORING STRESS HORMONES IN REHABILITATED AND CAPTIVE OTARIIDS

A

THESIS

Presented to the Faculty

of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

By

Lisa Petrauskas, B.S.

Fairbanks, Alaska

August 2005

ABSTRACT

Cortisol and corticosterone are the primary mammalian stress hormones released in response to a perceived stressor. Cortisol is rapidly metabolized in the blood, while corticosterone is the dominant product in fecal material. Radioimmunoassay procedures to measure fecal corticosterone and serum cortisol in California sea lions were validated, and adrenal response to surgical and non-surgical procedures was assessed. Other objectives included seasonal and behavioral variability in fecal corticosterone concentrations in captive Steller sea lions, as well as adrenal response to various stressors of a rehabilitated Steller sea lion.

There was a significant ($P \leq 0.05$) adrenal response for rehabilitated California sea lions that underwent minor invasive surgical procedures. The small sample size in this study allowed the identification of a correlation of season and behavior in three captive Steller sea lions. This study found that peak fecal corticosterone values reflected responses to acute stressors during rehabilitation for a Steller sea lion pup. Overall, fecal corticosterone was an adequate tool for monitoring stress non-invasively in California and Steller sea lions. In turn, the results indicate that California sea lions may be a suitable surrogate species to study the adrenal response to more invasive procedures that may be used in Steller sea lions.

TABLE OF CONTENTS

Signature page.....i

Title page.....ii

Abstract.....iii

Table of Contents.....iv

List of Figures.....vi

List of Tables.....viii

Acknowledgements.....ix

General Introduction.....1

Literature Cited.....8

Chapter 1. The effects of surgical and non-surgical procedures on fecal corticosterone concentrations in California sea lions.....13

Abstract.....13

Introduction.....14

Materials and Methods.....17

Animal selection.....17

Fecal sample extraction and preparation.....18

Serum sample preparation.....19

Assay validation.....20

Data analysis.....21

Results.....22

HPLC – RIA validation – serum cortisol and fecal corticosterone.....22

Fecal corticosterone experimental results....22

Discussion....22

Literature Cited....25

Figures and Tables....28

Chapter 2. Variation of fecal corticosterone concentrations in captive Steller sea lions in relation to season and behavior....39

Abstract....39

Introduction....40

Materials and Methods....43

Data analysis....45

Results....45

Discussion....46

Literature cited....48

Figures and Tables....52

Chapter 3. Non-invasive monitoring of stress hormone levels of a female Steller sea lion (*Eumetopias jubatus*) pup undergoing rehabilitation....56

Abstract....56

Brief Communication....57

Literature Cited....63

Figures and Tables....65

General Conclusions....67

LIST OF FIGURES

1-1 Displacement of serially diluted serum samples from female (A) and male (B) California sea lions to the cortisol standard curve (-○-).....28

1-2 Displacement of serially diluted fecal samples from female (A) and male (B) California sea lions to the corticosterone standard curve (-○-).....29

1-3 Quantitative assessment of the cortisol RIA for pooled female (A) and male (B) California sea lion serum.....30

1-4 Quantitative assessment of the corticosterone RIA for pooled female (A) and male (B) California sea lions feces.....31

1-5 Immunoreactivity of cortisol (-△-) and corticosterone (-▽-) in pooled female (A, $n = 5$) and male (B, $n = 5$,) California sea lion serum. ^{3}H-cortisol and ^{3}H-corticosterone (-●-) were added before HPLC as references. All units are expressed per 1ml fraction.....32

1-6 Immunoreactivity of cortisol (-△-) and corticosterone (-▽-) in pooled female (A, $n = 5$) and male (B, $n = 5$) California sea lion feces. ^{3}H-cortisol and ^{3}H-corticosterone (-●-) were added before HPLC as references. All units are expressed per 1ml fraction.....33

1-7 Mean (±SE) pre- and post-procedure fecal corticosterone concentrations for Group A (animals that were physically restrained for a blood sample), Group B (animals that underwent anesthesia without a surgical procedure), Group C (animals that underwent minor invasive surgery), and Group D (animals that underwent major invasive surgery). (*) denotes a significant difference ($P \leq 0.05$).....34

1-8 Mean (±SE) pre-procedure fecal corticosterone concentration for all California sea lions in this study based on health status at the time of the procedure. (*) denotes a significant difference.....35

2-1 Fecal corticosterone concentration (ng/g dry weight) for an adult male (a.) and two adult female (b. and c.) Steller sea lions during 2001-2004 based on Julian day. All years were combined and presented.....52

2-2 Linear regression and analysis of variance (ANOVA) for one adult male (a.) and two adult female (b., c.) Steller sea lions for mean weekly behavior score and fecal corticosterone concentration for samples collected 2001-2004.....53

2-3 Behavior score (-●-) and fecal corticosterone (-○-) concentrations (ng/g dry weight) for three Steller sea lions: (a.) SSL-01, (b.) SSL-02, (c.) SSL-03.....54

3-1 Mass of EJS-02-01 during 11 months of rehabilitation at ASLC.....65

3-2 Fecal corticosterone concentrations for a young Steller sea lion, EJS-02-01, during 11 months of rehabilitation at ASLC.....66

LIST OF TABLES

1-1 Number, age class, and sex of California sea lions used to evaluate the effects of restraint (Group A), anesthesia (Group B), minor surgery (Group C), and major surgery (Group D) on fecal corticosterone concentrations.....36

1-2 Fecal corticosterone concentration results from the Wilcoxon ranked sign test for Group A (animals that were physically restrained for a blood sample), Group B (animals that underwent anesthesia without a surgical procedure), Group C (animals that underwent minor invasive surgery), and Group D (animals that underwent major invasive surgery).....37

1-3 Summary description of fecal corticosterone concentrations (ng/g dry weight) at time zero (T_0) and the maximum fecal corticosterone concentrations within 48hr post-procedure (max.) for each animal.....38

2-1 Fecal corticosterone concentrations and behavior score for Steller sea lions.....55

ACKNOWLEDGEMENTS

I would like to thank all the people who helped me with sample collection, coordination, and support throughout the entire study. From The Marine Mammal Center: Michelle Caudle, Denise Greig, Dr. Frances Gulland, Dr. Marty Haulena, Lisa Phoenix, Shelbi Stoudt, Deb Wickham, and the numerous volunteers. From the Alaska SeaLife Center: Dennis Christen, Jen Dailer, Heather Down, Sara Dusage, Millie Gray, Lisa Hartman, Mike Illenberg, Tim Lebling, Kendall Mashburn, Elizabeth Moundalexis, Janelle Schuh, Carol Stephens, Dr. Pam Tuomi, and Lynn Turcotte.

In addition, I would like to thank my graduate committee for their support and guidance: Drs. Shannon Atkinson, Markus Horning, Jo-Ann Mellish, and Alan Springer.

Support for this project was provided by the Alaska SeaLife Center Steller Sea Lion Program with funding through the National Marine Fisheries Service and the Laboratory for Applied Biotelemetry & Biotechnology at Texas A&M University at Galveston with funding through the National Marine Fisheries Service, the North Pacific Marine Research Program and the Pollock Conservation Cooperative Research Center.

General Introduction

The physiological stress response generally refers to the coping mechanisms of an individual to perceived stressors. A stressor is defined as any disruption to homeostasis, which is the maintenance of a steady state within an organism, by physiological or behavioral feedback control mechanisms. Examples of stressors include, but are not limited to: establishing dominance, chase, capture, hunting, temperature extremes, and food or water deprivation (Nelson 2000). The typical coping mechanism to a perceived stressor was first described by Selye (1951) as the General Adaptation Syndrome: the animal detects the stressor and either copes with the stressor or terminates the stress response with the "exhaustion phase." The coping stage includes a release of glucocorticoids and/or catecholamines by the adrenal glands (Bauer *et al* 2001; Möstl and Palme 2002; Huber *et al* 2003). The mechanism of this response begins with the release of epinephrine from the adrenal medulla and norepinephrine from the sympathetic nervous system in response to a perceived stressor (Nelson 2000). Concurrently, the hypothalamic-pituitary-adrenal axis (HPA axis) is activated and corticotropin releasing factor (CRF) is released from the hypothalamus. The anterior pituitary gland then releases adrenocorticotropic hormone (ACTH) and prolactin. ACTH stimulates the adrenal cortex to release glucocorticoids, primarily cortisol and corticosterone in mammals. Levels of circulating epinephrine and norepinephrine return to normal shortly after the stressor is perceived to be removed, while glucocorticoid levels remain elevated for a longer period of time. Cortisol is rapidly metabolized within the bloodstream, while

corticosterone maintains its original state through metabolism and is excreted through fecal material (Norman and Litwack 1987).

There are six families of steroid hormones in mammals that are all biologically derived from cholesterol: estrogens, androgens, progestins, mineralocorticoids, glucocorticoids, and vitamin D (Norman and Litwack 1987). Glucocorticoids are synthesized in the adrenal cortex with targets that include liver, lymphoid cells, thymus gland, and kidney (Norman and Litwack 1987). Both acute and chronic stress result in glucocorticoid release to ensure the supply of energy to the individual via conversion of glycogen to energy rich glucose. Acute stress is defined as a controlled response to a stressor where the overall effect is not harmful (Goldstein 1995). Stress becomes detrimental, (i.e. chronic) when the organism responds to the stimuli in an excessive or uncontrolled manner (St. Aubin and Dierauf 2001). In addition to glucocorticoid release, adaptive responses to stress also include enhanced oxygen intake, memory and sensory function, with reciprocal decreased blood flow to non-essential areas, inhibition of digestion, growth, immune function, reproduction, and pain perception (Nelson 2000). Acute responses to stress are focused on the immediate survival of the animal, such that all future processes such as, energy storage as fat, production of gametes, and growth are suspended. These functions continue to remain in stasis during chronic stress which can lead to cell death and immunodeficiency. Other detrimental effects of chronic stress include muscle wasting, lack of calcium deposition, reproductive suppression, susceptibility to bruising, poor wound healing and dermal atrophy. Hypertension,

depression, sleep disturbance, memory impairment and cataracts can also occur (Norman and Litwack 1987; Millan *et al* 1996; Dhabhar and McEwen 1997; Silberman *et al* 2003).

Serum cortisol has been the preferred media for stress evaluation in animals, but is only a "snapshot" of the hormone level and may not truly monitor the overall "stressed" state of the animal. For instance, many species exhibit a diurnal rhythm in their glucocorticoid levels (Oki and Atkinson 2004), such that the timing of sampling can become an issue when comparing glucocorticoid concentrations from different groups of animals. Chronic stress in sheep has been shown to alter or abolish those diurnal rhythms, and therefore frequent blood sampling is required for accurate interpretation (Przkop *et al* 1985). Fecal corticosterone analysis is an alternative, non-invasive method to monitor glucocorticoids in organisms. Fecal samples are easily collected and provide an integrated response that represent longitudinal data over time, as opposed to serum cortisol levels that can change quickly from sample to sample (Möstl and Palme 2002). Fecal corticosterone assays have been validated to monitor adrenal activity in many animals: large terrestrial carnivores (Czekala *et al* 1994; Monfort *et al* 1996; Terio *et al* 1999; Wasser *et al* 2000; Turner *et al* 2002; de Villiers *et al* 2003); ruminants (Palme and Möstl 1996; Wasser *et al* 2000; Millspaugh *et al* 2002; Morrow *et al* 2002; Huber *et al* 2003); rodents (Harper and Austad 2000); birds (Ludders *et al* 2001; Wasser *et al* 2000); hares (Teskey-Gerstl *et al* 2000), and marine mammals (Wasser *et al* 2000; Mashburn and Atkinson 2004).

Quantification of stress in marine mammals has proven to be a significant challenge because of the inherent difficulty in obtaining baseline data from an unstressed

state (St. Aubin and Dierauf 2001). There are many natural and unnatural stressors that can affect marine mammals in the wild and in captivity. Some examples of natural stressors are predator avoidance, variable ocean conditions, diving, hunting for prey, establishment of social hierarchies, and reproduction. Human-related or unnatural stressors include: environmental contamination from industrial runoff, fisheries interactions, vessel traffic, sonar and low-frequency sound, and oil spills. In captivity, during rehabilitation or in aquaria, the chase, capture, handling, and sampling procedures can elicit a stress response (St. Aubin and Dierauf 2001). Therefore the question arises, when is stress in marine mammals harmful?

The family Otariidae consists of five species of sea lions and ten species of fur seals. The sea lions are a diverse group with separate geographical distributions and genus classifications. However, the California (*Zalophus californianus*) and Steller sea lion (*Eumetopias jubatus*) do overlap in habitat (Bonner 1994; Maniscalco *et al* 2004). California sea lions range from California, Oregon, Washington, and British Columbia, and recently there have been increased sightings in Alaska (Maniscaclo *et al* 2004). Steller sea lions range from Año Nuevo, in central California, north through Oregon, Washington, British Columbia, Alaska, and west to Russia and Japan. The primary overlap occurs in northern California (37° 07'N latitude and 122° 18'W longitude), Oregon, Washington, British Columbia, and eastern Alaska (60° 46'N and 170° 07'W).

The worldwide population of Steller sea lions has decreased dramatically since census counts began in the late 1960's. In 1990, the Steller sea lion was listed as threatened under the Endangered Species Act of 1973 (55 FR 49204). Studies into the

demographic and genetic diversity of Steller sea lions have shown that there are at least two distinct stocks separated at approximately 144° W longitude (Bickham *et al* 1996, Loughlin 1997). The Alaskan distribution of Steller sea lions has shown variable trends: counts of adult and juvenile Steller sea lions of the western stock have decreased approximately 85% since the 1970's (NMFS 1995). However in 2000, there was an increase in all trend sites (5.5%) and trend rookeries (6.8%) (Sease and Gudmundson 2002) and the eastern stock has been showing stable to increasing population numbers (Strick *et al* 1997). Due to the genetic and distribution variations between the two stocks, in 1997 the status of the western stock was re-classified as endangered, while the status of the eastern stock remained threatened (62 FR 30772).

In contrast, the California sea lion population has increased over the last century, with an average annual increase of 5% since the 1970's (Barlow *et al* 1995; NMFS 1997). Even though their population is healthy and productive, California sea lions are still protected under the Marine Mammal Protection Act (1972). The Act included a requirement to set up a response system for stranded marine mammals. As a result, in 1975, The Marine Mammal Center (TMMC) in Sausalito, CA was founded to rescue, rehabilitate, and research marine mammals, as well as to educate the public concerning marine mammals and the marine environment. TMMC is the largest rescue and rehabilitation organization in the United States. TMMC is responsible for over 600 miles of California coastline, with subsequent treatment of 600-800 sick and injured marine mammals each year. TMMC requires that external stimuli that can be perceived as stressful be kept at a minimum, mainly due to the link found between increased plasma

cortisol levels and increased herpesvirus mortality in Pacific harbor seals (*Phoca vitulina*) (Gulland *et al* 1999).

As interest and knowledge grows in marine mammal rehabilitation and research, there is an increased level of hands-on interaction and therefore potential for stress. Since there are many factors that can affect stress hormone levels in marine mammals, causal models are needed to generate predictions and guide experimental design and analysis (Fair and Becker 2000). Chronic restraint stress in rats increased basal levels of corticosterone (Bauer *et al* 2001), but because the animals were sacrificed 24 hours after restraint, it is unknown whether or not the corticosterone levels returned to baseline levels. Immobilization of African wild dogs did not provide cortisol concentrations that would be considered chronic after immobilization (70-82 min., de Villiers *et al* 1995). Palme *et al* (2000) found that a fecal cortisol metabolite in cattle increased significantly after transport then returned to baseline levels within 26-48hr.

Surgical procedures in marine mammals are more common due to the increased numbers of animals in rehabilitation programs and long-term monitoring of species in decline with various telemetry devices. Endoscopic procedures have been refined in bottlenose dolphins (*Tursiops truncates*), California sea lions (Dover and Van Bonn 2001) and sea otters (*Enhydra lutris*, Larson *et al* 2002). Chemical immobilization agents are listed for various species of cetaceans and pinnipeds (Haulena and Heath 2001). The only current method to asses the effect of these surgical and anesthetic procedures has been indirectly through survivorship rates (Williams and Siniff 1983; Mulcahy and Esler 1999; Dover and Van Bonn 2001; Hernandez-Divers 2001; Larson *et al* 2002).

A finer scale interpretation of the effects of these now common procedures on stress hormone concentrations is critical, particularly if protocols can be effectively modified to reduce stress and increase success rates. Efforts are being made in that direction with some species, such as the Steller sea lion. Mashburn and Atkinson (2004) found that the effect of isofluorane anesthesia on long-term, captive adult Steller sea lions had no effect on corticoid levels. The Satellite-Linked Life History Transmitter (LHX) Project (Horning and Mellish 2001) proposes to accurately determine the survival rate for wild juvenile Steller sea lions (1-4 years), which at present are estimated to have a survival rate to age 5 of only 50% (York 1994) based on a mathematical model. Early stages of the project will assess the effect of the implantation procedures, as well as the use of single and dual tag implants, on a surrogate, non-endangered species, the California sea lion.

This thesis focuses on the evaluation of non-invasive fecal steroid analysis to monitor stress response in California sea lions and Steller sea lions. The primary objectives are to:

1. Validate existing methods to measure cortisol as an indicator of stress in California sea lions.
2. Monitor long-term stress responses via non-invasive methods to ensure that rehabilitation procedures do not elicit a chronic stress response California and Steller sea lions.
3. Analyze the usefulness of California sea lions as a surrogate species for Steller sea lions for monitoring adrenal output.

Fecal corticosterone can be a useful tool to non-invasively monitor the complex reactions that result in a stress response. The ability to accurately measure stress hormone levels is critical to allow scientists to recognize and manage stress in marine mammals in captivity, animals in rehabilitation, and evaluate and monitor free-ranging populations.

LITERATURE CITED

Barlow, J., R.L. Brownell Jr., D.P. DeMaster, K.A. Forney, M.S. Lowry, S. Osmek, T.J. Ragen, R.R. Reeves, R.J. Small. 1995. U.S. Pacific marine mammal stock assessments. U.S. Dep. Commer., NOAA Tech. Memo. NMFSSWFSC219, 162p.

Bauer, M.E., P. Perks, S.L. Lightman, N. Shanks. 2001. Restraint stress is associated with changes in glucocorticoid immunoregulation. Physiology and Behavior. 73: 525-532.

Bickham, J.W., J.C. Patton, T.R. Loughlin. 1996. High variability for control-region sequences in a marine mammal: Implications for conservation and biogeography of Steller sea lions (*Eumetopias jubatus*). Journal of Mammalogy. 77(1): 95-108.

Bonner, N. 1994. Seals and sea lions of the world. Facts on File, Inc. New York.

Breazile, J.E. 1987. Physiologic basis and consequences of distress in animals. Journal of the American Veterinary Medical Association. 191(10): 1212-1215.

Breazile, J.E. 1988. The physiology of stress and its relationship to mechanisms of disease and therapeutics. Veterinary Clinics of North America: Food and Animal Practice. 4(3): 441-480.

Czekala, N.M., B.S. Durrant, L. Callison, M. Williams, S. Millard, 1994. Fecal steroid hormone analysis as an indicator of reproductive function in the cheetah. Zoo Biology. 13: 119-128

de Villiers, M.S., D.G.A. Meltzer, J. Van Heerden, M.G.L. Mills, P.R.K. Richardson, A.S. Van Jaarsveld. 1995. Handling-induced stress and mortalities in African wild dogs (*Lycaon pictus*). Proceedings of the Royal Society of London B. 262: 215-220.

Dhabhar, F.S. and B.J. McEwen. 1997. Acute stress enhances while chronic stress suppresses immune function "in vivo": a potential role for leukocyte trafficking. Brain Behavior and Immunity. 11: 286-306.

Dover, S.R. and W. Van Bonn. 2001. Flexible and rigid endoscopy in marine mammals, in *CRC Handbook of Marine Mammal Medicine*, Dierauf, L.A. and Gulland, F.M.D (ed.), CRC Press, Boca Raton, FL, 621-642.

Fair, P.A. and P.R. Becker. 2000. Review of stress in marine mammals. Journal of Aquatic Ecosystem Stress and Recovery. 7: 335-354.

Goldstein, D.S. 1995. Stress and science, in *Stress, Catecholamines and Cardiovascular Disease*, Goldstein, D.S. (ed.), Oxford University Press, Oxford, U.K., 3-55.

Gulland, F. M. D., M. Haulena, L.J. Lowenstine, C. Munro, P.A. Graham, J. Bauman, J. Harvey. 1999. Adrenal function in wild and rehabilitated Pacific harbor seals (*Phoca vitulina richardii*) and in seals with phocine herpesvirus-associated adrenal necrosis. Marine Mammal Science. 15(3): 810-827.

Harper, J.M. and S.N. Austad. 2000. Fecal glucocorticoids: a noninvasive method of measuring adrenal activity in wild and captive rodents. Physiological and Biochemical Zoology. 73(1): 12-22.

Haulena, M. and R.B. Heath. 2001. Marine mammal anesthesia, in *CRC Handbook of Marine Mammal Medicine*, Dierauf, L.A. and Gulland, F.M.D (ed.), CRC Press, Boca Raton, FL, 655-688.

Horning, M. and J.E. Mellish. 2001. Satellite-linked Mortality Transmitters in Steller Sea Lions: Assessing the effects of health status, foraging ability, and environmental variability of juvenile survival and population trends. A Proposal to the Steller Sea Lion Research Initiative. NMFS, 709 W. 9th St., 4th Floor, Juneau, AK, 99802-1668.

Huber, S., R. Palme, W. Arnold. 2003. Effects of season, sex, and sample collection on concentrations of fecal cortisol metabolites in red deer (*Cervus elaphus*). General and Comparative Endocrinology. 130: 48-54.

Huber, S., R. Palme, W. Zenker, E. Möstl. 2003. Non-invasive monitoring of the adrenocortical response in red deer. Journal of Wildlife Management. 67(2): 258-266.

Larson, S., R. Jameson, J. Bodkin, M. Staedler, P. Bentzen. 2002. Microsatellite DNA and mitochondrial DNA variation in remnant and translocated sea otter (*Enhydra lutris*) populations. Journal of Mammalogy. 83(3): 893-906.

Loughlin, T.R. 1997. Using the phylogeographic method to identify Steller sea lion stocks. Molecular Genetics of Marine Mammals, Spec. Pub. 3:159-171.

Ludders, J.W., J.A. Langenberg, N.M. Czekala, H.N. Erb. 2001. Fecal corticosterone reflects serum corticosterone in Florida sandhill cranes. Journal of Wildlife Diseases. 37(3): 646-652.

Maniscalco, J.M., K. Wynne, K.W. Pitcher, M.B. Hanson, S.R. Melin, S. Atkinson. 2004. The occurrence of California sea lions (*Zalophus californianus*) in Alaska. Aquatic Mammals. 30(3): 427-433.

Mashburn, K.L. and S. Atkinson. 2004. Evaluation of adrenal function in serum and feces of Steller sea lions (*Eumetopias jubatus*): influences of molt, gender, sample storage, and age on glucocorticoid metabolism. General and Comparative Endocrinology. 136(3): 371-381.

Merrick, Richard L. and T.R. Loughlin. 1997. Foraging behavior of adult female and young-of-the-year Steller sea lions in Alaskan waters. Canadian Journal of Zoology. 75: 776-786.

Millan, S., M.I. Gonzalez-Quijano, M. Giordano, L. Soto, A.I. Martín, A. Lopez-Calderon. 1996. Short and long restraint differentially affect humoral and cellular immune functions. Life Sciences. 59: 1431-1442.

Millspaugh, J.J., B.E. Washburn, M.A. Milanick, J. Beringer, L.P. Hansen, T.M. Meyer. 2002. Non-invasive techniques for stress assessment in white-tailed deer. Wildlife Society Bulletin. 30(3): 899-907.

Mizoguchi, K., M. Yuzurihara, A. Ishige, H. Sasaki, H. Chui, T. Tabira. 2001. Chronic stress differentially regulates glucocorticoid negative feedback in rats. Psychoneuroendocrinology. 26: 443-459.

Monfort, S.L., K.L. Mashburn, B.A. Brewer, S.R. Creel. 1996. Evaluating adrenal activity in African wild dogs (*Lycaon pictus*) by fecal corticosteroid analysis. Journal of Zoo and Wildlife Medicine. 29(2): 129-133.

Morrow, C.J., E.S. Kolver, G.A. Verkerk, L.R. Matthews. 2002. Fecal glucocorticoid metabolites as a measure of adrenal activity in dairy cattle. General and Comparative Endocrinology. 126: 229-241.

Möstl, E. and R. Palme. 2002. Hormones as indicators of stress. Domestic Animal Endocrinology. 23: 67-74.

National Marine Fisheries Service (NMFS). 1995. Status review of the United States Steller sea lion, *Eumetopias jubatus*, population. U.S. Dep. Commer., NOAA, National Marine Mammal Laboratory, AFSC, 7600 Sand Point Way NE, Seattle, WA 98115. 61p.

National Marine Fisheries Service (NMFS). 1997. Investigation of Scientific Information on the Impacts of California Sea Lions and Pacific Harbor Seals on Salmonids and on the Coastal Ecosystems of Washington, Oregon, and California. U.S. Dep. Commer., NOAA Tech. Memo. NMFS-NWFSC-28, 172p.

National Marine Fisheries Service (NMFS). 2000. A Discussion Paper on Potential Interactions Between Steller Sea Lions and the BSAI and GOA Pacific Cod Fisheries. NMFS, Alaska Region. Division of Protection Resources. Alaska Fisheries Science Center, 55p.

Nelson, R.J. 2000. An Introduction to Behavioral Endocrinology. 2nd ed. Sinauer Associates, Inc. Sunderland, MA.

Norman, A.W. and G. Litwack. 1987. Hormones. Academic Press, Inc. San Diego, CA.

Oki, C. and S. Atkinson. 2004. Diurnal patterns of cortisol and thyroid hormones in the harbor seal (*Phoca vitulina*) during summer and winter seasons. General and Comparative Endocrinology. 136(2): 289-297.

Palme, R. and E. Möstl. 1996. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. 1st International Symposium on Physiologie and Ethology of Wild and Zoo Animals, Berlin, September 18-21.

Palme, R., C. Robia, W. Baumgartner, E. Möstl. 2000. Transport stress in cattle as reflected by an increase in faecal cortisol metabolite concentrations. Veterinary Record. 146. 108-109.

Przkop, F., E. Stupnicka, E. Wolinska-Witort, K. Mateusiak, B. Sadowsi, E. Domanski. 1985. Changes in circadian rhythm and suppression of the plasma cortisol level after prolonged stress in the sheep. Acta Endocrinologica. 110: 540-545.

Sease, J.L. and C.J. Gudmundson. 2002. Aerial and land-based surveys of Steller sea lions (*Eumetopias jubatus*) from the western stock of Alaska, June and July 2001 and 2002. U. S. Dep. Commer., NOAA Tech. Memo. NMFS-AFSC-131, 54p.

Selye, H. 1951. The general adaptation syndrome and the diseases of adaptation. The American Journal of Medicine. 10(5): 549-555.

Silberman, D.M., M.R. Wald, A.M. Genaro. 2003. Acute and chronic stress exert opposing effects on antibody responses associated with changes in stress hormone regulation of T-lymphocyte reactivity. Journal of Neuroimmunology. 144: 53-60.

St. Aubin, D.J. and L.A. Dierauf. 2001. Stress and Marine Mammals, in *CRC Handbook of Marine Mammal Medicine*, Dierauf, L.A. and Gulland, F.M.D (ed.), CRC Press, Boca Raton, FL, 253-269.

Strick, M., L. Fritz, J.P. Lewis, D.C. McAllister. 1997. Aerial and ship-based surveys of Steller sea lions (*Eumetopias jubatus*) in Southeast Alaska, the Gulf of Alaska and Aleutian Islands during June and July 1994. U.S. Dep. Commer., NOAA Tech. Memo. NMFS-AFSC-71.

Terio, K.A., S.B. Citino, J.L. Brown. 1999. Fecal cortisol metabolite analysis for noninvasive monitoring of adrenocortical function in the cheetah (*Acinonyx jubatus*). Journal of Zoo and Wildlife Medicine. 30(4): 484-491.

Teskey-Gerstl, A., E. Bamberg, T. Steineck, R. Palme. 2000. Excretion of corticosteroids in urine and faeces of hares (*Lepus europaeus*). Journal of Comparative Physiology B: Biochemical, Systemic and Environmental Physiology. 170: 163-168.

Turner, J.W., P. Tolson, N. Hamad. 2002. Remote assessment of stress in white rhinoceros (*Ceratotherium simum*) and black rhinoceros (*Diceros bicornis*) by measurement of adrenal steroids in feces. Journal of Zoo and Wildlife Medicine. 33(3): 214-221.

Wasser, S.K., K.E. Hunt, J.L. Brown, K. Cooper, C.M. Crockett, U. Bechert, J.J. Millspaugh, S. Larson, S.L. Monfort. 2000. A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. General and Comparative Endocrinology. 120: 260-275.

York, A.E. 1994. The population dynamics of Northern sea lions, 1975-1985. Marine Mammal Science. 10(1): 38-51.

Chapter 1. The effects of surgical and non-surgical procedures on fecal corticosterone concentrations in California sea lions [1]

ABSTRACT

We validated existing methods of steroid hormone analysis in marine mammals to enable the quantification of stress in rehabilitated California sea lions. In order to establish methods for monitoring stress that would be broadly applicable to both captive and free-ranging populations of California sea lions; procedures with both serum and feces as substrates were validated. Four experimental categories (Group A: restraint blood draw [n=9], Group B: anesthesia, no surgery [n=10], and Group C: anesthesia and minor invasive surgery [n=10]) were selected from routine rehabilitation procedures, while Group D: anesthesia and major invasive surgery [n=5]) was selected by the veterinary staff. Feces were collected opportunistically up to 72hr prior and 72hr post procedure for corticosterone analysis. A serum sample was obtained during each categorical procedure in order to validate serum as a substrate for steroid hormone analysis. Both fecal corticosterone and serum cortisol radioimmunoassays (RIA) were validated with standard methods including High Pressure Liquid Chromatography (HPLC). Results indicated substantial individual variation in fecal corticoid responses. Sea lions that were compromised at the time of the procedure had significantly higher fecal corticosterone concentrations than animals that were considered healthy at the time of their respective procedure ($P = 0.002$). There was no significant difference between

[1] Prepared for submission in *General and Comparative Endocrinology* as Petrauskas, L., S. Atkinson, F. Gulland, and M. Horning. The effects of surgical and non-surgical procedures on fecal corticosterone concentrations in California sea lions.

fecal corticosterone concentrations before and after the procedures of Groups A, B, or D. However, there was a significant ($P < 0.05$) difference in fecal corticosterone concentration before and after minor invasive surgery (Group C). Fecal corticosterone levels may be a suitable indicator of long term corticoid production, and may be used as a non-invasive tool for the assessment of stress in rehabilitated sea lions subject to surgical procedures.

INTRODUCTION

Research and rehabilitation of marine mammals is on the rise, and with that comes an increased level of hands-on interaction and potential for stress. Stress physiology is the study of perturbations that upset physiological homeostasis and the efforts to re-establish that balance (Sapolsky 1992). A stressor can be physical, psychological, acute, or chronic.

Corticotropin-releasing hormone (CRH) is released from the hypothalamus in response to a perceived stressor. CRH stimulates the anterior pituitary gland to release adrenocorticotropic hormone (ACTH), which in turn stimulates the adrenal cortex to release glucocorticoids. Glucocorticoids and catecholamines (epinephrine and norepinephrine), which are released from the adrenal medulla, together mediate most of the changes that form the stress response. Glucocorticoids are commonly referred to as corticoids, stress hormones, or adrenal activity. Major glucocorticoids in mammals are steroid hormones, primarily cortisol and corticosterone.

Handling wild animals in a rehabilitation setting may elicit a stress response. Marine mammal rehabilitation programs are taking in much higher numbers than in the past due to increased awareness by the public. With technological advancements in veterinary medicine, most marine mammals that enter rehabilitation undergo some type of procedure. These procedures may be non-invasive, mildly invasive, or a major invasive procedure. Surgery on marine mammals with the use of chemical immobilization agents and endoscopic procedures have been refined in recent years (Dover and Van Bonn 2001; Haulena and Heath 2001; Larson *et al.* 2002). There is also the need for long-term monitoring of species in decline with various telemetry devices that have the ability to last years. The only current method to asses the effect of these surgical, anesthetic, and telemetric procedures has been indirectly and short-term through survivorship to the procedures themselves (Williams and Siniff 1983; Mulcahy and Esler 1999; Dover and Van Bonn 2001; Hernandez-Divers 2001; Larson *et al.* 2002).

Stress hormone levels need to be investigated in order to accurately interpret the effects of these increasingly common invasive procedures, especially if protocols can be modified to effectively reduce stress and increase survivability. Limited research to date includes the study of corticoid response to isofluorane anesthesia in captive, adult Steller sea lions (*Eumetopias jubatus*, Mashburn and Atkinson 2004) and response to various acute stressors and surgical procedures in a single rehabilitated Steller sea lion pup (Petrauskas *et al.* in review). Most studies of telemetry device implantation in wild and captive animals only investigated behavioral and physical responses to the immediate procedures. Such studies have been performed in European badgers (*Meles meles*, Ågren

et al. 2000), North American river otters (*Lontra Canadensis*, Hernandez-Divers *et al.* 2001), and sea otters (*Enhydra lutris*, Williams and Siniff 1983). In one instance, post-release mortality was studied in harlequin ducks (*Histrionicus histrionicus*) that were implanted with abdominal radio transmitters (Mulcahy and Esler 1999).

The ongoing Satellite-Linked Life History Transmitter (LHX) Project (Horning and Mellish 2001) proposes to accurately determine the survival rate for juvenile Steller sea lions (1-4 years), which at present are estimated to have a survival rate of only 50% between weaning and the age of 5 years (York 1994) based on a mathematical model. California sea lions (*Zalophus californianus*) will be used as a surrogate species for initial tests of the effect of the implantation procedures. They overlap from northern California (37° 07'N latitude and 122° 18'W longitude), Oregon, Washington, British Columbia, to eastern Alaska (60° 46'N and 170° 07'W) (Bonner 1994; Maniscalco *et al.* 2004).

The objectives of this study were to:

1. Evaluate the use of non-invasive fecal steroid analysis to monitor the stress response in rehabilitated California sea lions that underwent a range of surgical procedures.
2. Validate existing methods to measure cortisol as an indicator of stress in California sea lions.

MATERIALS AND METHODS

Animal selection

Four experimental categories were designed from existing, routine procedures in rehabilitated California sea lions at The Marine Mammal Center (TMMC) Sausalito, CA. The primary reason for admission for all experimental animals was trauma, particularly abscesses. Other common reasons for admission were *Leptospirosis sp.*, pneumonia, domoic acid toxicity, and malnutrition. Health status of all animals in this study was based on the veterinary record at the time of the procedure as compromised or non-compromised.

Group A (n=9) consisted of animals that were physically restrained for a blood sample (Table 1-1). The typical protocol includes utilizing herding boards, a towel to cover the animal's eyes, and a person to restrain and straddle the animal. Restraint included the application of light pressure to the shoulders and head of the animal, but did not require chemical immobilizing agents.

Group B (n=10) included animals that underwent isofluorane anesthesia for non-surgical purposes (e.g. x-ray, Table 1-1). All animals in Group B were not physically restrained, but were chemically sedated with atropine, metatomadine, and telazol, then placed on isofluorane anesthesia for 5-40 minutes. Anesthesia was subsequently reversed with atipamezole. Procedures included: radiographs (n=7), wound examination (n=1), or ophthalmic examination (n=2).

Group C (n=10) animals underwent isofluorane anesthesia for minor invasive surgical procedures (Table 1-1). Animals were chemically sedated and reversed as

described above for Group B. Isofluorane anesthesia duration was from 15-150 minutes. Procedures included abscess lances with drain placements (n = 3) and third eyelid suturing to heal corneal ulcerations (n = 7).

Group D (n=5) animals were subjected to isofluorane anesthesia for major invasive surgical procedures (Table 1-1). Animals were anesthetized and reversed as described as above. Isofluorane anesthesia duration ranged from 80-105 minutes. Procedures included Life History Transmitter (LHX) implantation (n = 4) and laparoscopy (n = 1). Sea lions that underwent LHX implantation were selected by TMMC veterinary staff and were non-compromised at the time of surgery. The transmitters were inserted through an incision into the abdominal cavity and placed between the caudal sternum manubrium and the pubic bones.

Stress levels associated with invasive and implant surgeries were assessed through fecal corticosterone levels. Fecal samples for all groups were collected opportunistically up to 72hrs prior and 72hrs post procedure. A blood sample was obtained during each categorical procedure in order to validate serum as a substrate for steroid hormone analysis.

Fecal sample extraction and preparation

To extract corticosterone from fecal material, all samples were fully mixed, aliquoted (~5 g), loaded onto a rotary evaporator (Speed-Vac Plus, SC110A; Savant Instruments, Holbrook, NY), and dried without heat. Dried fecal samples were crushed into powder and 0.025 g (± 0.001) was weighed and extracted as previously described

(Monfort *et al* 1996; Mashburn and Atkinson 2004). Methanol (MeOH) extractant (100 µl) was aliquoted into polypropylene tubes, dried under forced air and reconstituted in 900 µl buffer for a final 1:10 dilution. Sample dilutions were stored frozen at -20°C until radioimmunoassay (RIA).

A double antibody RIA kit using ^{125}I (MP Biomedicals, Costa Mesa, CA) was used for corticosterone analysis. Radioactivity was determined using a gamma counter (Gamma C12, Diagnostic Products, Los Angeles, CA). Standard curves of RIAs were log-logit transformed in order to read the hormone concentrations off the standard curve (Rodbard 1974). Values from the RIA were corrected for dilution, extraction efficiency, weight of fecal material extracted, and expressed as ng/g dry weight. The RIAs were performed according to manufacturer's instructions with the exception that all volumes were halved and an additional standard was added to the curve (i.e., one-half the lowest standard) to increase sensitivity. Manufacturer cross-reactivity with other steroids was as follows: desoxycorticosterone (0.34%), testosterone (0.10%), cortisol (0.05%), aldosterone (0.02%) and <0.01% for all other steroids tested. Inter-assay coefficient of variation for the assay control was 18.3 % (n = 22 for all samples assayed). Intra-assay coefficients of variation were <5% and assay sensitivity was 13.8 ng/tube.

Serum sample preparation

A solid phase RIA kit (Diagnostic Products, Los Angeles, CA) for cortisol was used for unextracted, undiluted California sea lion serum cortisol validation. Radioactivity was determined using a gamma counter (Gamma C12, Diagnostic Products,

Los Angeles, CA). Standard curves of RIAs were log-logit transformed in order to read the hormone concentrations off the standard curve (Rodbard 1974). The RIAs were performed per manufacturer instructions with the exception that all volumes were halved. Manufacturer cross-reactivity data are as follows: prednisolone (76.0%), 11-deoxycortisol (11.4%), prednisone (2.3%), cortisone (0.98%), corticosterone (0.94%), tetrahydrocortisol (0.34%), 11-deoxycorticosterone (0.26%), aldosterone (0.03%), progesterone and pregnenolone (0.02%), flumethasone (0.017%), and 0.01% or below for all other steroids tested. Inter-assay coefficient of variation for the assay control was 12.1% (n=6 for all samples assayed). Intra-assay coefficients of variation were <5% and assay sensitivity was 0.63 μg/tube.

Assay validation

Each RIA was validated with standard methods including high pressure liquid chromatography (HPLC). A serum pool was created for both male and female sea lions to validate the cortisol assay. Serial dilutions (neat to 1:1024) of both sex pools yielded displacement parallel to the standard curve (Fig. 1-1). A fecal pool for both males and females was created similarly to validate the corticosterone assay. Serial dilutions (neat to 1:1024) of both sex pools yielded displacement parallel to the standard curve (Fig. 1-2). Recovery of added cortisol (range 0.5 – 25 μg/dL) was 101.2% (SD = 9.01; CV = 8.9%) for females ($y = 1.65 + 1.04x$, $r^2 = 0.99$) and 107.5% (SD = 14.02; CV = 13.0%) for males ($y = 0.25 + 1.02x$, $r^2 = 0.99$) (Fig. 1-3). Recovery of added corticosterone (range 12.5 – 500 μg/dL) was 107.1% (SD = 17.88; CV = 16.7%) for females ($y = -5.24 +$

1.05x, $r^2 = 1.0$) and 100.6% (SD = 11.74; CV = 11.7%) for males ($y = 1.49 + 1.02x$, $r^2 = 1.0$) (Fig. 1-4). For HPLC (Varian ProStar 210/215, Varian, Walnut Creek, CA), both male and female serum and fecal pools were spiked with tritiated cortisol and corticosterone as reference tracers. The samples were eluted through a reverse phase C-18 column using an organic/aqueous solvent gradient of 20-80% MeOH/H_2O to 100% MeOH over 80min with a flow rate of 1ml/min, with each milliliter collected as a separate fraction. Following HPLC, 100µl of each fraction was placed in a scintillation counter to determine the fraction in which the cortisol and corticosterone were eluted. The remaining portions of each fraction were divided into equal aliquots of 450µl each, dried down, then reconstituted in the appropriate amount of buffer. Each aliquot was then run in both cortisol and corticosterone RIAs to determine immunoreactivity present in each fraction.

Data analysis

Differences in fecal corticosterone concentration between compromised and non-compromised animals were analyzed using the Mann-Whitney rank sum test.

Changes in fecal samples for all groups were analyzed using the Wilcoxon Signed Rank Test, with the null hypothesis of no change in fecal corticosterone concentrations before and after the varying procedures rejected at $P<0.05$.

RESULTS

HPLC – RIA validation - serum cortisol and fecal corticosterone

Serum aliquots run in a cortisol RIA co-eluted in association with the cortisol peak and represented 99.7% and 77.4% of the total mass eluted for females and males, respectively (Fig. 1-5). Female and male serum aliquots run in a corticosterone RIA co-eluted in association with the corticosterone peak and represented 88.0% and 38.8%, respectively of the total mass eluted (Fig. 1-5). Fecal aliquots run in a cortisol RIA did not yield an immunoreactive peak associated with cortisol for either sex (Fig. 1-6). Fecal aliquots run in a corticosterone RIA co-eluted in association with the corticosterone peak and represented 66.8% and 31.2% of the total mass eluted for females and males, respectively (Fig. 1-6).

Fecal corticosterone experimental results

Compromised sea lions of all groups had significantly higher fecal corticosterone concentrations at the time of their individual procedures (Fig. 1-8; $P = 0.002$).

Groups A, B and D did not exhibit a significant difference in fecal corticosterone concentrations pre- and post-procedures (Fig. 1-7; Table 1-2) whereas Group C differed significantly (Table 1-2; $P \leq 0.05$).

DISCUSSION

HPLC results indicated that both corticosterone and cortisol are present in measurable quantities in serum (Fig. 1-5), while corticosterone is the only measurable

glucocorticoid in feces (Fig. 1-6). For this study, a commercially available corticosterone RIA kit was used for fecal corticosterone analysis. Fecal samples are easily collected and provide an integrated response that represents longitudinal data over a period of time as opposed to serum stress hormone analysis where the concentrations can change quickly from sample to sample (Möstl and Palme 2002).

Overall, 65% of the California sea lions in this study were deemed non-compromised at the time of their individual procedures. Twenty-three of 34 sea lions displayed an increased adrenal response to the particular procedure that they underwent (Table 1-3). Of those 23 animals, 14 were considered non-compromised at the time of the procedure. This suggests that the animals experienced an acute response to the various perceived stressors in terms of the experimental categories designed for this study. Thirty of the 34 sea lions were released back into the wild successfully. These data are similar to the results found in Petrauskas *et al.* (in review) where a Steller sea lion pup rehabilitated with similar procedures was released successfully.

Groups A, B, C, and D were selected to assess the response to an increasing invasiveness index ranging from baseline physical restraint for a blood sample to major invasive surgical procedures.

Seven of the nine animals Group A did show an overall stress response, but there was not a significant difference between fecal corticosterone concentrations before and after the procedure (Table 1-2). Wild loggerhead sea turtles (*Caretta caretta*) and southern elephant seal pups (*Mirounga leonina*) showed marked acute adrenocortical responses in serum in response to physical restraint and blood sampling (Gregory *et al*

1996; Engelhard *et al* 2002), but fecal analysis was not conducted. Eight out of the 10 animals in Group A were released successfully.

There was considerable variation in fecal corticosterone concentration for the sea lions in Group B. Six of the 10 animals did elicit a stress response, but there was no overall significant difference between fecal corticoid levels before and after the procedures (Table 1-2). Adrenal hormones vary within and among animals and may respond differently based on daily rhythm, wild vs. domesticated animals, sex differences, varying population densities (Turner and Bagnara 1976). Similar results were found with field handling and anesthesia techniques for radiocollaring African wild dogs (*Lycaon pictus*, Creel *et al* 1997) such that non-surgical anesthetic procedures were not stressful and did not provoke an immunosuppressive response.

Seven of the 10 animals in the minor invasive category of Group C did elicit an acute stress response, with significantly higher fecal corticosterone concentrations post-procedure (Table 1-2). This response could have been due to the longer isofluorane anesthesia exposure, increased invasiveness of the procedures or longer healing time required. Glucocorticoids are essential to the anti-inflammatory response (Breazile 1988) and were most likely released in larger quantities to counter the minor surgical procedures, and Mashburn and Atkinson (2004) found no response to isofluorane anesthesia and corticoid concentrations in Steller sea lions. Nine out of ten of the animals in this group were released, indicating that the acute stress of the procedure did not affect survival and ultimate release back into the wild.

There was a steroid response in 3 of the 5 animals of Group D, but it was not a significant response (Table 1-2), most likely due to the small sample size. All five animals were released successfully. These results indicate that while there may be an acute stress response to the introduction of an abdominal telemetry device, the response was not chronic enough to adversely affect long-term survivability.

Overall, fecal corticosterone analysis appears to be a practical and reliable method to non-invasively monitor the stress response to varying surgical and non-surgical techniques. This method allows for the determination of adrenal activity over a longer period of time than is feasible with serum samples. Combined with a reduced handling requirement for sample collection, this is an ideal method to monitor wild animals in rehabilitation or recovering from a surgical procedure.

LITERATURE CITED

Ågren, E.O., L. Nordenberg, T. Mörner. 2000. Surgical implantation of radiotelemetry transmitters in European badgers (*Meles meles*). Journal of Zoo and Wildlife Medicine. 31(1): 52-55.

Braezile, J.E. 1988. The physiology of stress and its relationship to mechanisms of disease and therapeutics. Veterinary Clinics of North America: Food Animal Practice. 4(3): 441-480.

Creel, S., N.M. Creel, S.L. Monfort. 1997. Radiocollaring and stress hormones in African wild dogs. Conservation Biology. 11(2): 544-548.

Dover, S.R. and W. Van Bonn. 2001. Flexible and rigid endoscopy in marine mammals, in *CRC Handbook of Marine Mammal Medicine*, Dierauf, L.A. and Gulland, F.M.D (ed.), CRC Press, Boca Raton, FL, 621-642.

Engelhard, G.H., S.M.J.M. Brasseur, A.J. Hall, H.R. Burton, P.J.H. Reijnders. 2002. Adrenocortical responsiveness in southern elephant seal mothers and pups during lactation and the effect of scientific handling. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology. 172: 315-328.

Gregory, L.F., T.S. Gross, A.B.Bolten, K.A. Bjorndal, L.J. Guillette, Jr. 1996. Plasma corticosterone concentrations associated with acute captivity stress in wild loggerhead sea turtles (*Caretta caretta*). General and Comparative Endocrinology. 104: 312-320.

Haulena, M. and R.B. Heath. 2001. Marine mammal anesthesia, in *CRC Handbook of Marine Mammal Medicine*, Dierauf, L.A. and Gulland, F.M.D (ed.), CRC Press, Boca Raton, FL, 655-688.

Hernandez-Divers, S.M., G.V. Kollias, N. Abou-Madi, B.K. Hartup. 2001. Surgical technique for intra-abdominal radiotransmitter placement in north American river otters (*Lontra Canadensis*). Journal of Zoo and Wildlife Medicine. 32(2): 202-205.

Horning, M. and J.E. Mellish. 2001. Satellite-linked Mortality Transmitters in Steller Sea Lions: Assessing the effects of health status, foraging ability, and environmental variability of juvenile survival and population trends. A Proposal to the Steller Sea Lion Research Initiative. NMFS, 709 W. 9th St., 4th Floor, Juneau, AK, 99802-1668.

Larson, S., R. Jameson, J. Bodkin, M. Staedler, P. Bentzen. 2002. Microsatellite DNA and mitochondrial DNA variation in remnant and translocated sea otter (*Enhydra lutris*) populations. Journal of Mammalogy. 83(3): 893-906.

Maniscalco, J.M., K. Wynne, K.W. Pitcher, M.B. Hanson, S.R. Melin, S. Atkinson. 2004. The occurrence of California sea lions (*Zalophus californianus*) in Alaska. Aquatic Mammals. 30(3): 427-433.

Mashburn, K. and S. Atkinson. 2004. Evaluation of adrenal function in serum and feces of Steller sea lions (*Eumetopias jubatus*): influences of molt, gender, sample storage, and age on glucocorticoid metabolism. General and Comparative Endocrinology. 136(3): 371-381.

Möstl, E. and R. Palme. 2002. Hormones as indicators of stress. Domestic Animal Endocrinology. 23: 67-74.

Mulcahy, D.M. and D. Esler. 1999. Surgical and immediate postrelease mortality of harlequin ducks (*Histrionicus histrionicus*) implanted with abdominal radio transmitters with percutaneous antennae. Journal of Zoo and Wildlilfe Medicine. 30(3): 397-401.

Rodbard, D. 1974. Statistical quality control for radioimmunoassay and immunoradiometric assays. Clinical Chemistry. 20(10). 1255-1270.

Sapolsky, R.M. 1992. Neuroendocrinology of the stress-response, in *Neuroendocrinology of the stress response*. Becker JB; Breedlove SB; Crews D (ed.) MIT Press, Cambridge, USA, 287-324.

Turner, C.D. and J.T. Bagnara. 1976. General Endocrinology. 6th ed. W.B.Saunders Company. Philadelphia, PA.

Williams, T.D. and D.B. Siniff. 1983. Surgical implantation of radiotelemetry devices in the sea otter. Journal of the American Veterinary Medical Association. 183: 1290-1291.

York, A.E. 1994. The population dynamics of Northern sea lions, 1975-1985. Marine Mammal Science. 10(1): 38-51.

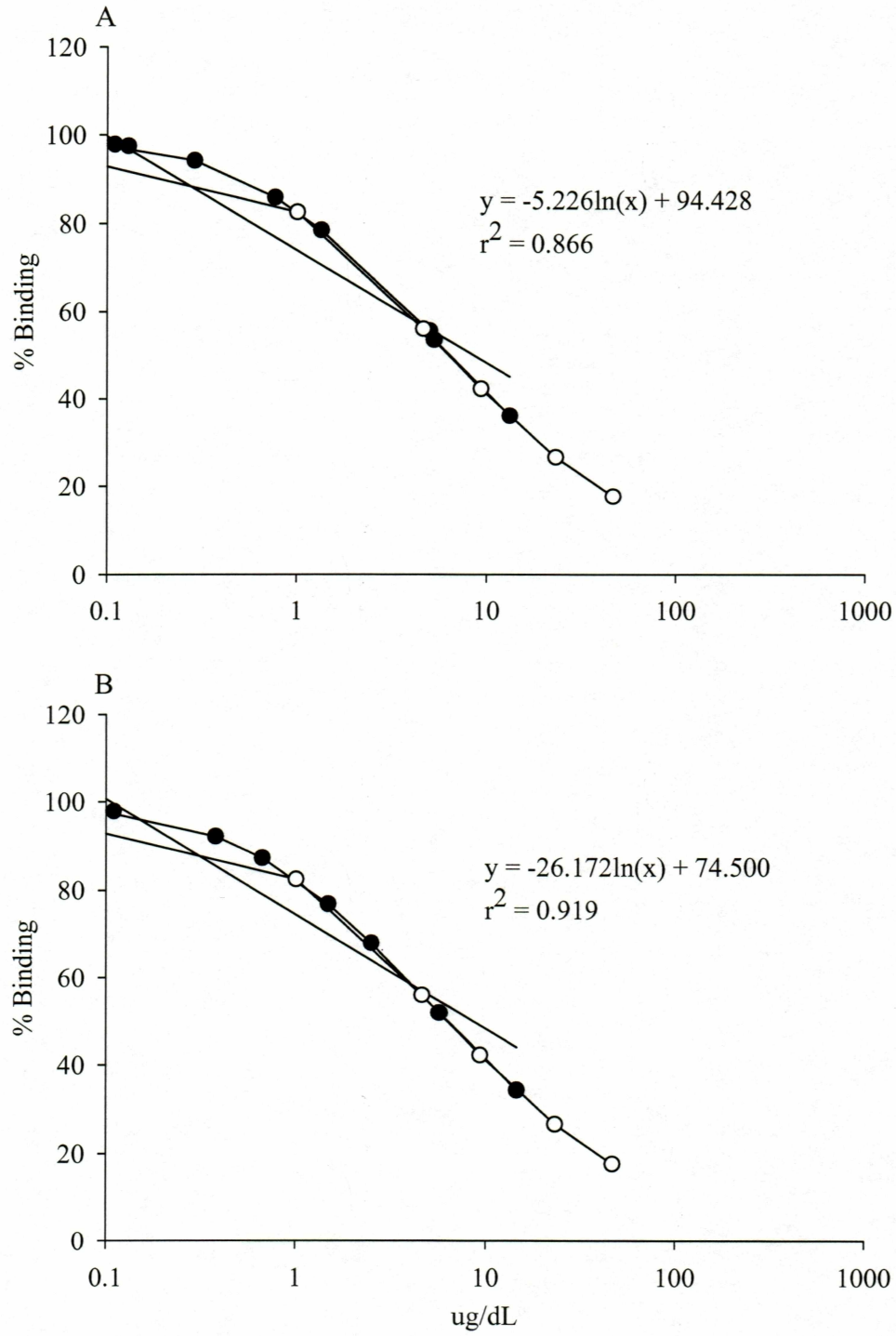

FIG. 1-1. Displacement of serially diluted serum samples from female (A) and male (B) California sea lions to the cortisol standard curve (-○-).

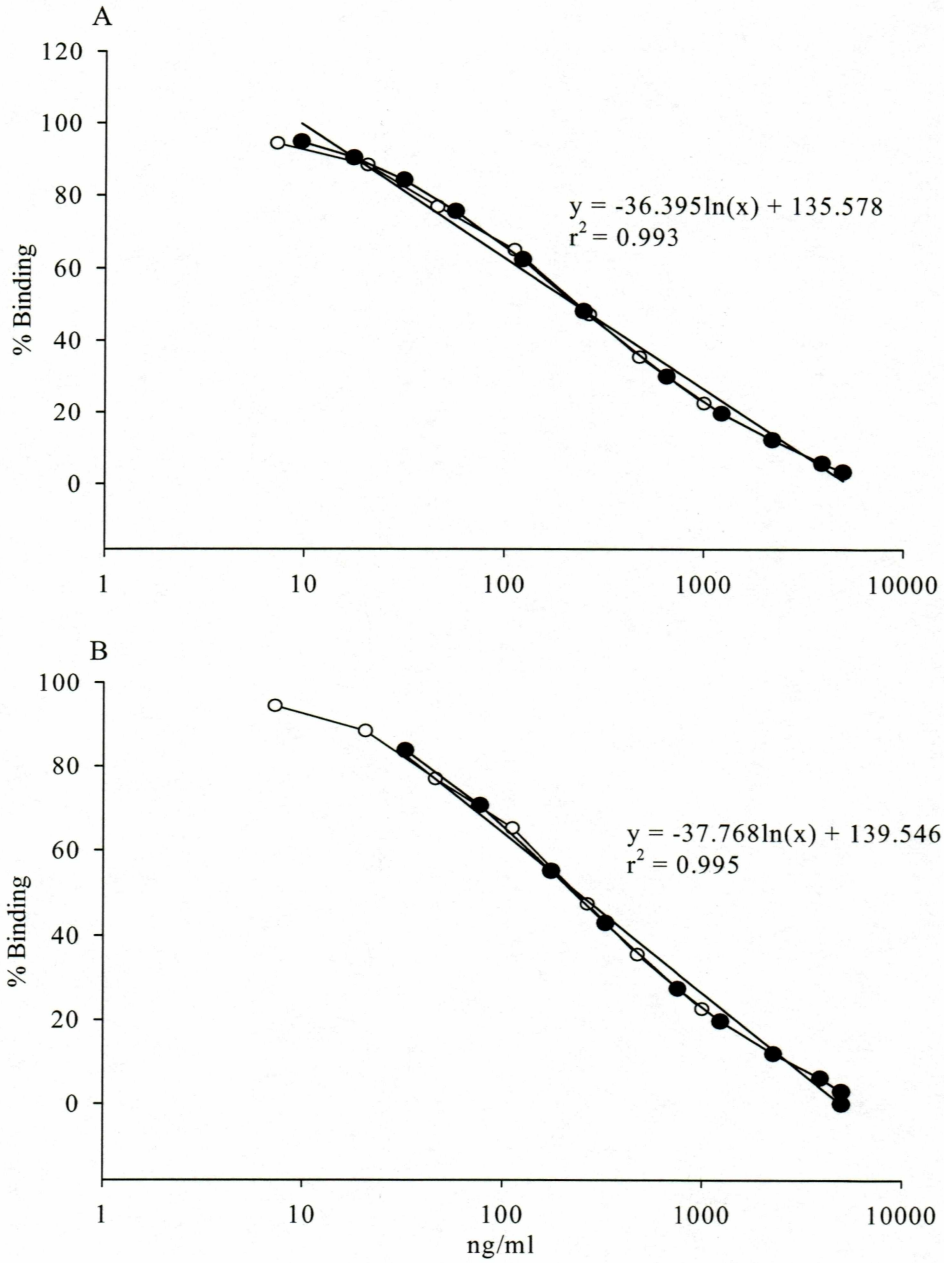

FIG. 1-2. Displacement of serially diluted fecal samples from female (A) and male (B) California sea lions to the corticosterone standard curve (-○-).

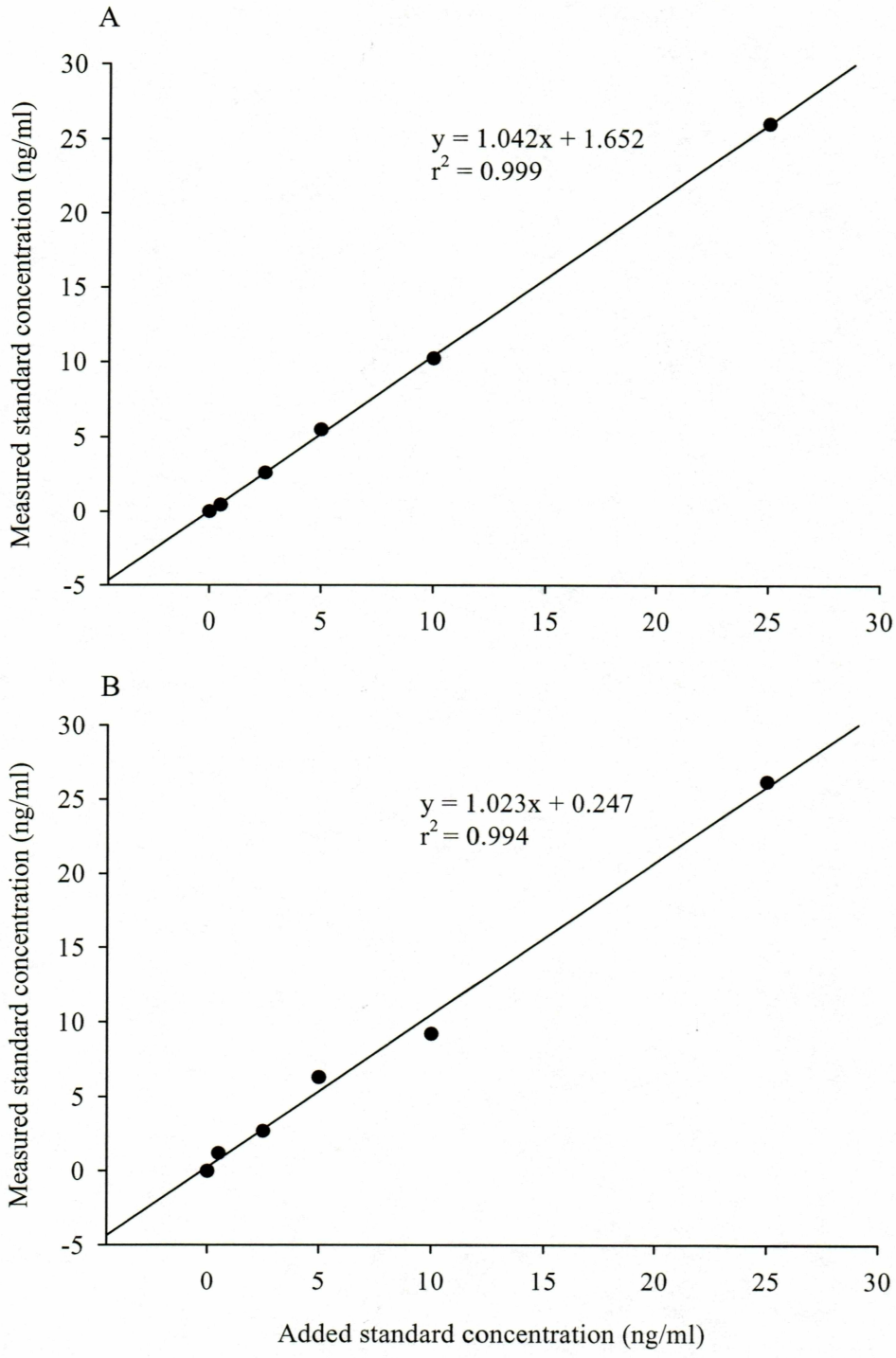

FIG. 1-3. Quantitative assessment of the cortisol RIA for pooled female (A) and male (B) California sea lion serum.

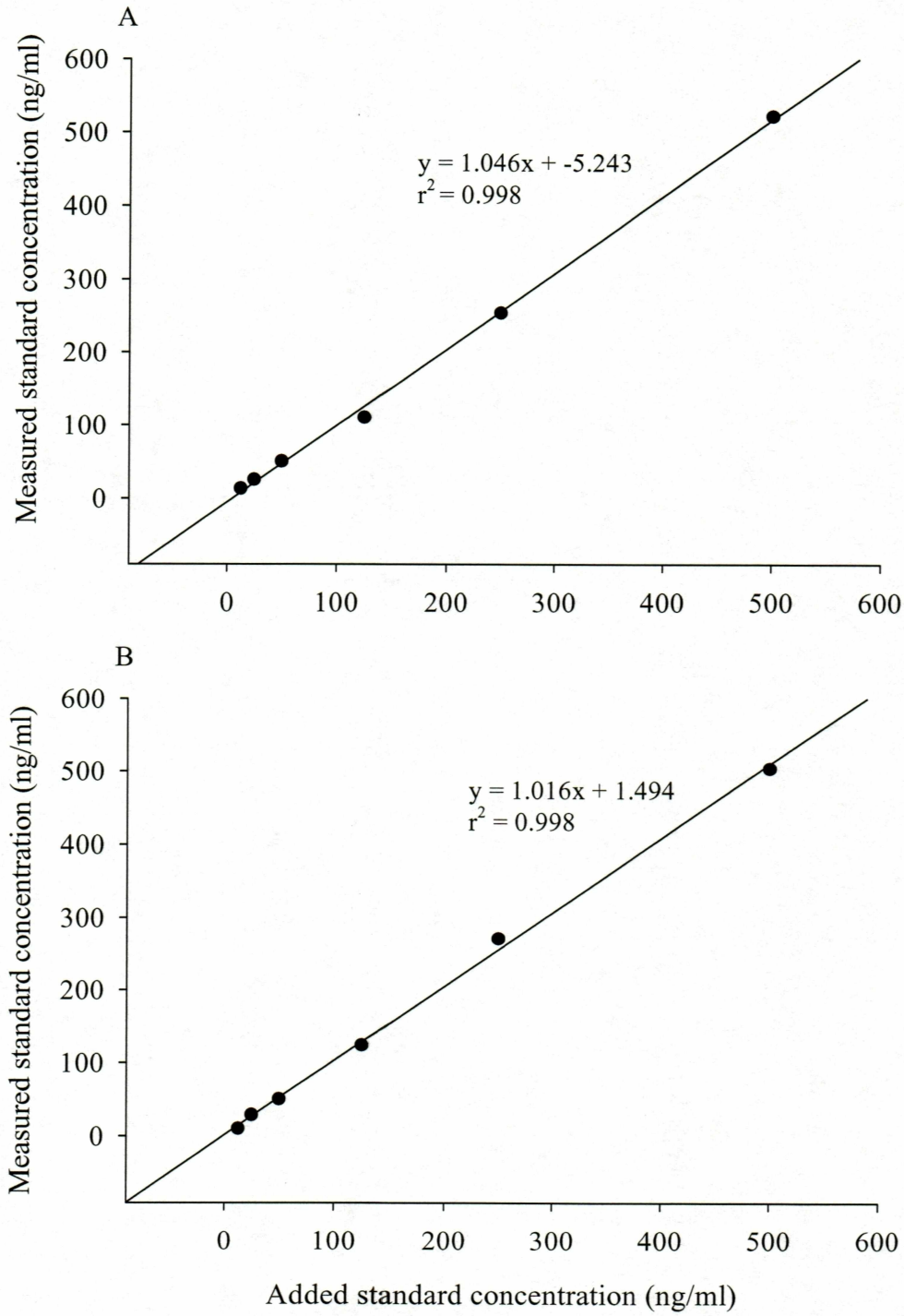

FIG. 1-4. Quantitative assessment of the corticosterone RIA for pooled female (A) and male (B) California sea lions feces.

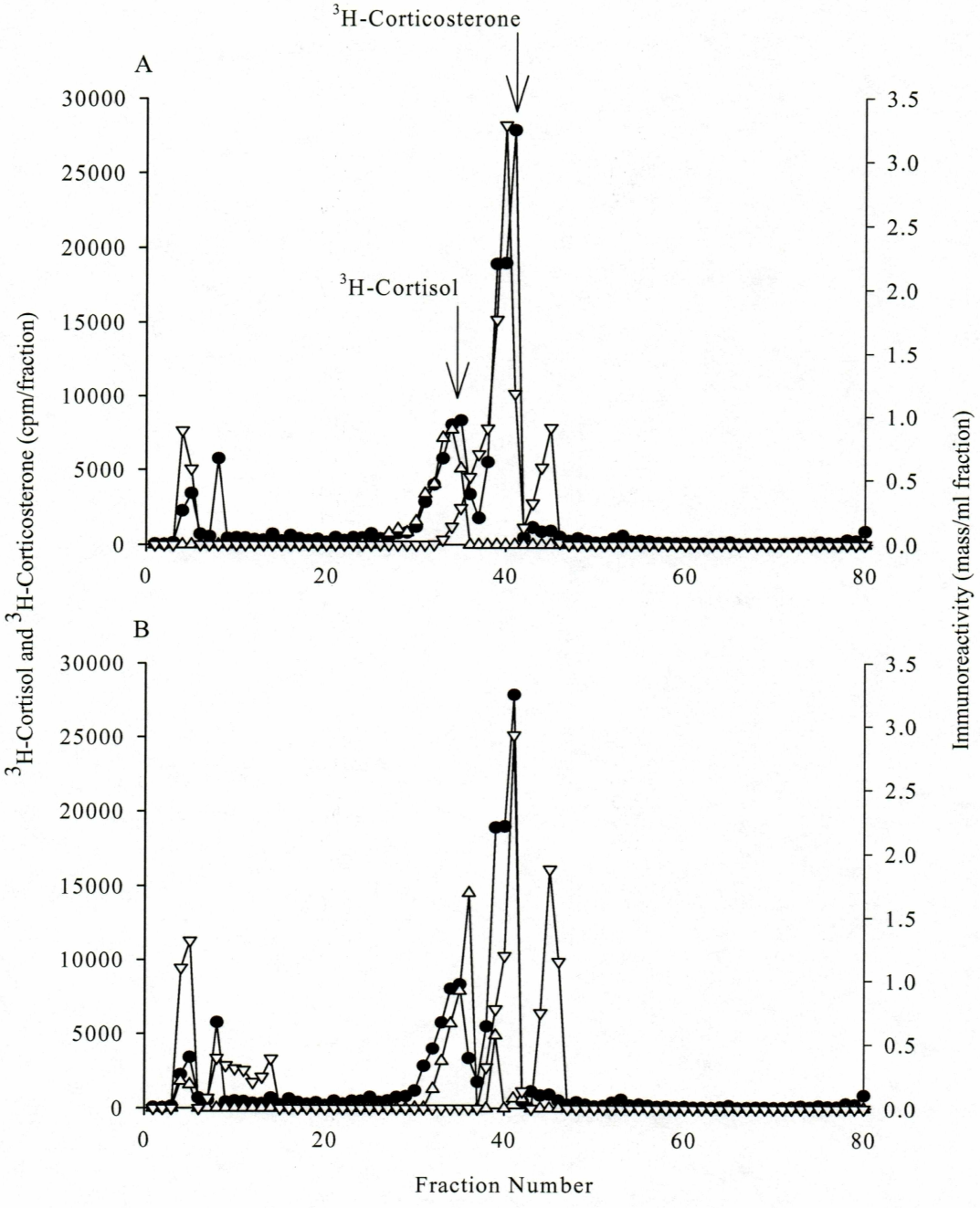

FIG. 1-5. Immunoreactivity of cortisol (-△-) and corticosterone (-▽-) in pooled female (A, $n = 5$) and male (B, $n = 5$) California sea lion serum. ^{3}H-cortisol and ^{3}H-corticosterone (-●-) were added before HPLC as references. All units are expressed per 1ml fraction.

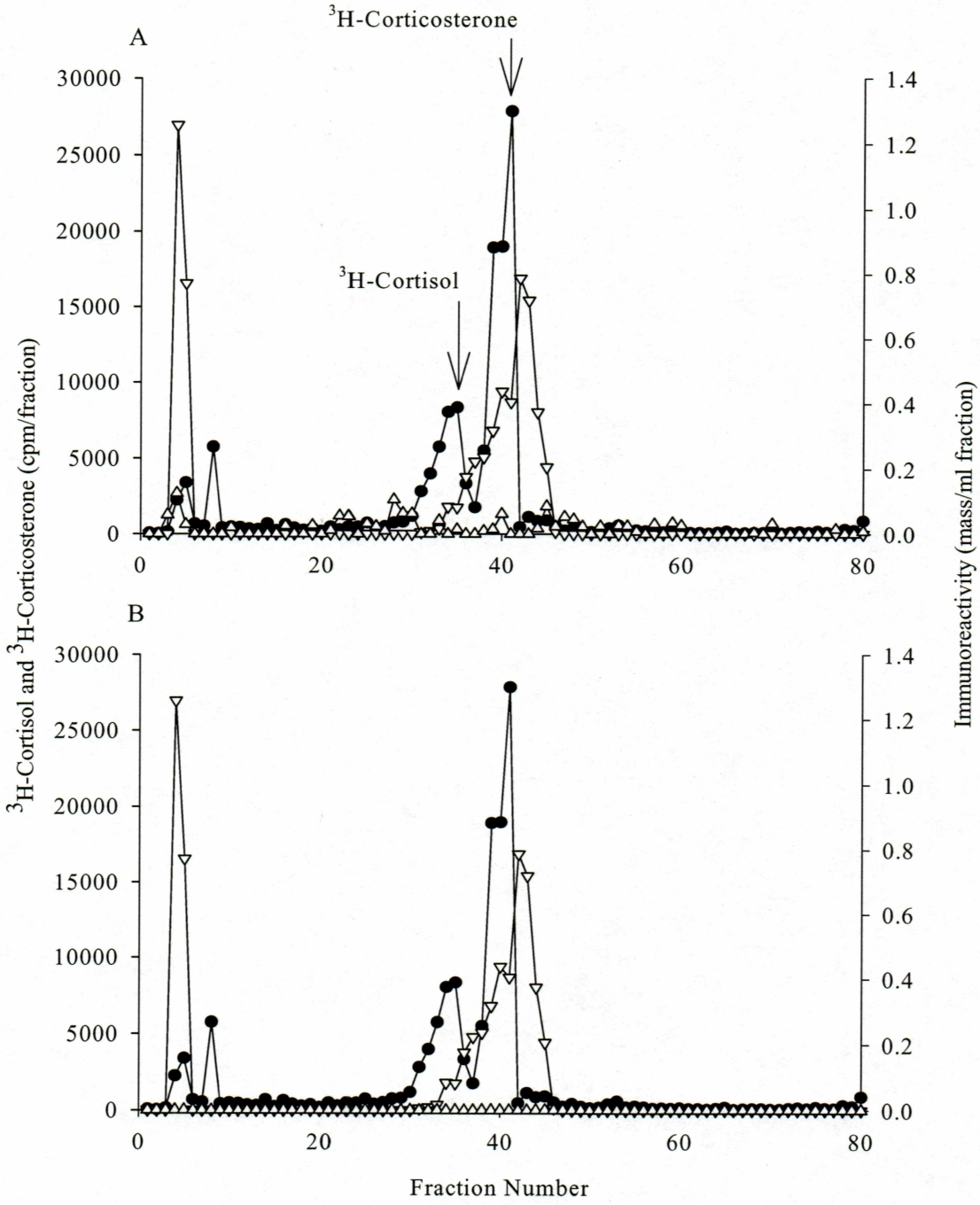

FIG. 1-6. Immunoreactivity of cortisol (-△-) and corticosterone (-▽-) in pooled female (A, $n = 5$) and male (B, $n = 5$) California sea lion feces. ^{3}H-cortisol and ^{3}H-corticosterone (-●-) were added before HPLC as references. All units are expressed per 1ml fraction.

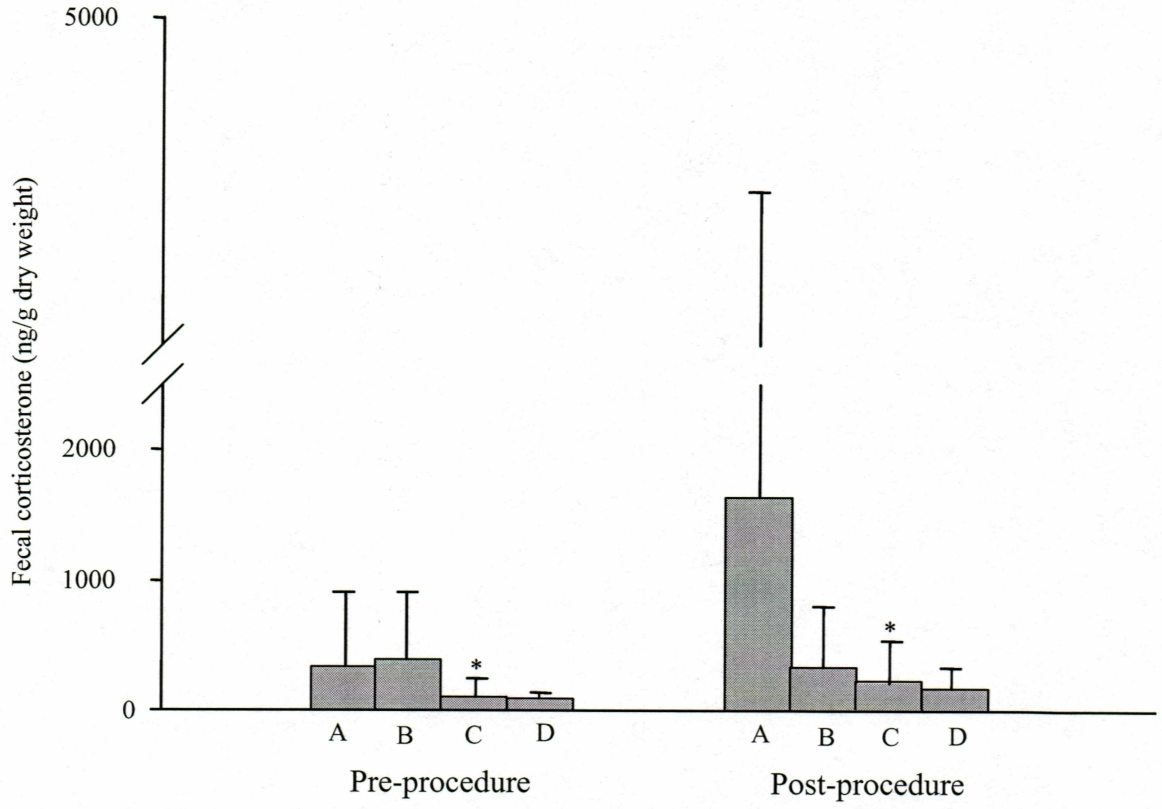

FIG. 1-7. Mean (±SE) pre- and post-procedure fecal corticosterone concentrations for Group A (animals that were physically restrained for a blood sample), Group B (animals that underwent anesthesia without a surgical procedure), Group C (animals that underwent minor invasive surgery), and Group D (animals that underwent major invasive surgery). (*) denotes a significant difference ($P \leq 0.05$).

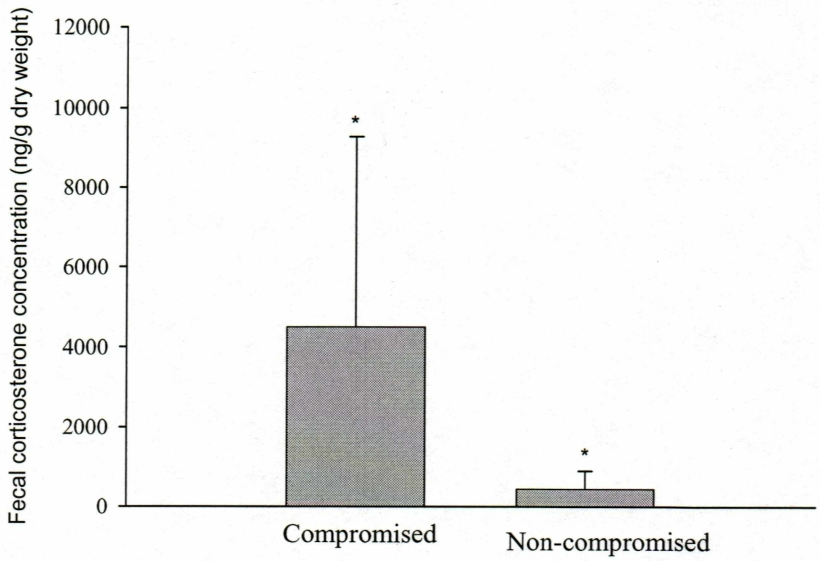

FIG. 1-8. Mean (±SE) pre-procedure fecal corticosterone concentration for all California sea lions in this study based on health status at the time of the procedure. (*) denotes a significant difference (P=0.002).

TABLE 1-1. Number, age class, and sex of California sea lions used to evaluate the effects of restraint (Group A), anesthesia (Group B), minor surgery (Group C), and major surgery (Group D) on fecal corticosterone concentrations.

Group	Male				Female		
	Yearling	Juvenile	SubAdult	Adult	Yearling	Juvenile	Adult
A	1	2	1	1	1	2	1
B	5	0	0	1	2	1	1
C	3	3	0	0	4	0	0
D	0	0	3	1	0	0	1

TABLE 1-2. Fecal corticosterone concentration results from the Wilcoxon ranked sign test for Group A (animals that were physically restrained for a blood sample), Group B (animals that underwent anesthesia without a surgical procedure), Group C (animals that underwent minor invasive surgery), and Group D (animals that underwent major invasive surgery).

Group	Mean (±SE) pre-procedure samples	Mean (±SE) post-procedure samples	*P*-Value
A	3378.5 ± 5741.36	16331.0 ± 31019.08	>0.05
B	3933.2 ± 5192.61	3385.2 ± 4689.86	>0.05
C	1042.0 ± 1412.65	2150.2 ± 3262.35	<0.05
D	929.4 ± 440.55	1722.4 ± 1598.28	>0.05

TABLE 1-3. Summary description of fecal corticosterone concentrations (ng/g dry weight) at time zero (T_0) and the maximum fecal corticosterone concentrations within 48hr post-procedure (max.) for each animal.

ID#	T_0	max.	Δ	Compromised (y/n)	ID#	T_0	max.	Δ	Compromised (y/n)
Group A					Group C				
5595	228.1	1007.3	+	n	5302	130.6	720.1	+	n
5527	290.3	222.7	+	n	6007	1702.5	368.3	-	n
5255	5.8	354.1	+	n	5278	107.9	405.5	+	n
5499	498.1	1112.7	+	n	5292	177.5	14831.7	+	n
5289	1019.6	388.1	-	n	5231	117.2	358.4	+	n
6139	17965.4	37202.6	+	y	5286	207.4	126.5	-	n
5312	1636.0	1110.9	-	y	5249	152.3	336.5	+	n
5319	5686.5	43521.7	+	y	5547	4743.7	2193.3	-	y
5318	5286.1	28202.4	+	y	5556	5213.7	5923.9	+	y
					5265	169.4	421.8	+	y
Group B					Group D				
5221	514.5	515. 8	n.c.	n	6018	202.5	1718.6	+	n
6022	1376.7	8493.5	+	n	6053	167.3	492.8	+	n
5493	393.1	1550.5	+	n	5406	372.9	2974.6	+	n
5814	421.6	477.2	n.c.	n	6039	248.0	112.4	-	n
5844	682.0	1153.8	+	n	6116	1087.3	96.3	-	n
5270	5712.0	1136.7	-	y					
5259	3132.5	6983.7	+	y					
5275	127.9	270.6	+	y					
5294	302.9	200.1	-	y					
5906	3942.2	4461.7	+	y					

Note: Group A: animals that were physically restrained for a blood sample; Group B: animals that underwent anesthesia without a surgical procedure; Group C: animals that underwent minor invasive surgery; Group D: animals that underwent major invasive surgery. (+) indicates an increase in adrenal output from T_0, and (-) indicates a decrease in adrenal output from T_0. Compromised animals were determined by the veterinary staff at the time of procedure and n.c. indicates no discernable change in fecal corticosterone concentration pre- and post-procedures.

Chapter 2. Variation of fecal corticosterone concentrations in captive Steller sea lions in relation to season and behavior [2]

ABSTRACT

Little information is available regarding adrenal activity of Steller sea lions in relation to season and behavior. The objective of this study was to test for seasonal changes in fecal corticosterone concentrations and potential relationships to behavioral scoring in captive Steller sea lions. For this study, fecal samples were obtained opportunistically over a three year period (Sept. 2001-04) from three adult (1 male, 2 female), reproductively intact, long-term captive Steller sea lions housed at the Alaska SeaLife Center (Seward, Alaska). Daily behavior scores were also recorded. There was a significant difference between seasons and fecal corticosterone concentrations for the male (SSL-01) and one of the females (SSL-03; $P \leq 0.05$), but seasonal differences varied among the individuals. Overall, fecal corticosterone concentrations differed between the two females ($P < 0.001$). There was a significant difference between behavior score and fecal corticosterone concentrations for the male and one female (SSL-03).

[2] Prepared for submission in *Aquatic Mammals* as Petrauskas, L., M. Horning, and S. Atkinson. Variation of fecal corticosterone concentrations in captive Steller sea lions in relation to season and behavior.

INTRODUCTION

Stress physiology is the study of perturbations that upset the physiological balance and the mechanisms that re-establish homeostasis in the body. Possible stressors to an organism can commonly include: changes in environment or food resources, season, social status, and behavior. A stressor can cause an acute or chronic stress response. Acute stress is defined as a controlled response to a stressor where the overall effect is not harmful (Goldstein 1995). Stress becomes detrimental, or chronic, when a state of distress causes the organism to respond to the stimuli in an excessive or uncontrolled manner over a prolonged period of time (St. Aubin and Dierauf 2001).

When an organism perceives a stressor, the hypothalamus releases corticotrophin-releasing hormone (CRH). The CRH that is released, in turn stimulates the anterior pituitary gland which releases adrenocorticotropic hormone (ACTH). ACTH then stimulates the adrenal cortex to release glucocorticoids. The stress response is then mediated by the glucocorticoids as well as catecholamines (epinephrine and norepinephrine) that are released from the adrenal medulla. The primary glucocorticoids in mammals are cortisol and corticosterone.

There are four accepted methods for measuring corticoids in mammals. Cortisol and corticosterone can be measured in serum and provides data corresponding to the moment of collection. Cortisol can be measured in saliva, which requires restraint in animals not trained to give those samples (Bushong *et al* 2000). Cortisol and corticosterone can also be measured in urine, but can require changes in housing, restraint, or training for collection (Teskey-Gerstl *et al.* 2000). Serum, saliva, and urine

therefore can be difficult or invasive samples to obtain with collection procedures contributing or confounding the end result. In contrast, fecal samples integrate corticosterone concentrations over longer periods of time, are a non-invasive collection, can be used in wild, captive or rehabilitating mammals, and have been validated for use in measuring adrenal activity in Steller sea lions (*Eumetopias jubatus*, Mashburn and Atkinson 2004).

Glucocorticoids are involved in the general homeostasis of energy as well as in the acute and chronic stress responses (Kenagy and Place 2000), but may fluctuate as a function of season. Extreme weather conditions can disrupt breeding if animals are unable to cope physiologically (Romero *et al.* 2000). Male muriquis (*Brachyteles arachnoids*) fecal cortisol concentrations were elevated during copulatory periods that coincided with the normal dry season that followed an unusually heavy rainy season (Strier *et al.* 1999). Seasonal and reproductive state also influences glucocorticoid levels. Male Arctic passerines (*Zonotrichia leucophrys gambelii*) had higher plasma glucocorticoids than females during the breeding season (Astheimer *et al.* 1994). Corticosterone in breeding Arctic passerines and redpolls (*Acanthis flammea*) of both sexes were significantly higher than their non-breeding counterparts (Astheimer *et al.* 1994; Wingfield *et al.* 1994). Plasma corticosterone in male chipmunks (*Tamias amoenus*) peaked immediately following the mating period (Place and Kenagy 2000), and plasma cortisol changes with breeding and molting seasons of wild harbor seals (*Phoca* vitulina) (Gardiner and Hall 1997). However, seasons do not appear to influence serum cortisol in wild and semi-domesticated bottlenose dolphins (*Tursiops* truncates, St. Aubin

et al. 1996) and captive harbor seals (Gardiner and Hall 1997). Although, Oki and Atkinson (2004) found that the diurnal pattern of cortisol secretion was abandoned in the summer in captive harbor seals.

Behavioral endocrinology is the study of how the general physiology of hormones may alter behavior by increasing the chance that a certain behavior will occur when a particular stimulus is present, and in return, how behavior can influence hormones (Nelson 2000). Behavior is most commonly thought of as an output, or a response to a stressor, but the reciprocal can occur; behavior can affect hormone levels. Primates and humans that have lost battles, from territorial contests to chess matches, have shown reduced testosterone levels immediately following those losses (Nelson 2000). Accessibility to a variety of toy and feeding enrichment activities positively influenced the behavioral and physiological responses in brown capuchins (*Cebus paella*). An increase in normal behaviors corresponded to a decrease in plasma cortisol (Boinski *et al.* 1999). Unpredictable events, like severe storms, increased secretion of corticosterone in white-crowned sparrows (*Zonotrichia leucophrys*), which influenced their behavior through altered migration patterns (Wingfield and Ramenofsky 1997). Rhesus monkeys (*Macaca mulatto*) that did not have control, or lost control, over high intensity noise had significantly increased plasma cortisol levels, increased aggressive behavior, and less social contact with other animals (Hanson *et al.* 1976). Rats that could not predict electric shock treatments through a signal exhibited stress-induced pathologies, such as stomach ulcerations, increased plasma corticosterone, decreased body weight, decreased food and

water intake, and increased defecation rates, whereas the rats that could predict the shock did not (Weiss 1970).

In recent years captive Steller sea lions have provided basic metabolic, physiologic, and veterinary information (Sato *et al.* 1998; Rea *et al.* 2000; Hunt *et al.* 2004; Mashburn and Atkinson 2004). One study monitored the impact of rehabilitation on growth and fecal corticosterone concentrations for a Steller sea lion pup (Petrauskas *et al.* in review). Another study validated fecal corticosterone as a method to monitor adrenal activity in Steller sea lions (Mashburn and Atkinson 2004). However, little information is available regarding adrenal activity in relation to season and behavior in large marine mammals. The primary objective of this study was to monitor the fecal corticosterone concentration in captive Steller sea lions in relation to season and behavior. Monitoring adrenal activity in captive Steller sea lions may provide a baseline for free-ranging studies due to the copious amount of known fecal samples that can be collected, and can be correlated to the age of the animal, season, sex, and behavior of the individual.

MATERIALS AND METHODS

Fecal samples were obtained from three adult (1 male, SSL-01: 2 females, SSL-02 & SSL-03), reproductively intact, captive Steller sea lions housed under ambient conditions at the Alaska SeaLife Center (ASLC) in Seward, Alaska. All animals were eight years of age at the beginning of sample collection. Behavior was based on the mean of appetite, energy, attention, sociability, and enrichment scores in the husbandry

record. Samples were collected opportunistically from September 2001 through September 2004 and frozen at -20°C until extraction.

To extract corticosterone from fecal material, all samples were fully mixed, aliquoted (~5 g), loaded onto a rotary evaporator (Speed-Vac Plus, SC110A; Savant Instruments, Holbrook, NY), and dried without heat. Dried fecal samples were crushed into powder and 0.025 g (± 0.001) was weighed and extracted as previously described (Monfort *et al.* 1996; Mashburn and Atkinson 2004; Petrauskas *et al. in review*). Methanol extractant (100 µl) was aliquoted into polypropylene tubes, dried under forced air, reconstituted in 400 µl buffer for a final 1:4 dilution. Sample dilutions were stored frozen at -20°C until radioimmunoassay (RIA).

A double antibody RIA kit (MP Biomedicals, Costa Mesa, CA) previously validated for use with Steller sea lion fecal extracts (Mashburn and Atkinson 2004) was used for corticosterone analysis. Standard curves of RIAs were log-logit transformed in order to read the hormone concentrations off the standard curve (Rodbard 1974). Values from the RIA were corrected for dilution, extraction efficiency, weight of fecal material extracted, and expressed as ng/g dry weight. RIAs were performed according to manufacturer's instructions with the exception that all volumes were halved and an additional standard was added to the curve (i.e., one-half the lowest standard) to increase sensitivity. Manufacturer cross-reactivity with other steroids was as follows: desoxycorticosterone (0.34%), testosterone (0.10%), cortisol (0.05%), aldosterone (0.02%) and less than 0.01% for all other steroids tested. Interassay coefficients of

variation for two separate assay controls were 14.9 and 14.5% (n = 10 for all samples). Intra-assay coefficients of variation were <5% and assay sensitivity was 12.7 ng/tube.

Data analysis

A repeated measures design was used to determine if the difference between season and behavior was related to corticosterone concentrations. Fecal corticosterone and time of year was tested for significance using the linear-circular rank-correlation test. Differences in corticosterone concentration and social status were tested using the Mann-Whitney rank sum test. Linear regression with one-way analysis of variance (ANOVA) and the Mann-Whitney rank sum test were used to test the relationship between behavior and corticosterone concentration. Significance was based at a P-value ≤ 0.05.

RESULTS

There was a significant relationship between Julian day and fecal corticosterone concentration for SSL-01 and SSL-03 (Fig. 2-1).

There was a significant difference between the weekly mean behavior score (appetite, attention, energy, sociability, and enrichment) of animals with elevated fecal corticosterone concentrations and non-elevated concentrations, as well as a significant linear relationship between behavior score and fecal corticosterone concentration for the male ($y = -6.15x + 3.13$; Fig. 2-2a) and one of the females (SSL-03; $y = -4.85x + 3.05$; Fig. 2-2c). However, for female SSL-02, there was a significant difference between

behavior score when compared with elevated (≥ 500 ng/g) and non-elevated (< 500 ng/g) fecal corticosterone concentration (Table 2-1).

DISCUSSION

Fecal corticosterone concentrations varied with season and behavior, with individual seasonal patterns. The breeding season of Steller sea lions begins in May when males return to the rookery to defend and maintain prime breeding sites. Reproductive condition was based on season and it has been well documented that glucocorticoids vary based on reproductive status (Astheimer *et al.* 1994; Wingfield *et al.* 1994; Theodorou and Atkinson 1998; Strier *et al.* 1999; Place and Kenagy 2000). The male had the highest corticosterone concentrations in the spring following into the summer (Fig. 2-1). Elevated corticosterone levels for the females were at the end of the summer for SSL-02 and the end of winter for SSL-03 (Fig. 2-1). The captive female Steller sea lions at ASLC began their estrus in July of all study years as indicated by a pink and swollen vulva.

Behavior was based on the mean of appetite, energy, attention, sociability, and enrichment scores in the husbandry record. SSL-01 and SSL-03 (Fig. 2-2a, 2-2c; Table 2-1) displayed decreased behavior scores with increased fecal corticosterone. Immediately prior to and during the breeding season, male Steller sea lions are engulfed in territorial battles that would potentially decrease appetite, attention, sociability and enrichment. Typically, female Steller sea lions do not engage in the same territorial battles as the males, but when stressed do have to cope with perceived stressors on the rookery, such as territorial battles going on around them, or weather and surf conditions.

Agonistic and aggressive interactions amongst social and co-operatively breeding species can be a chronic stressor (Sands and Creel 2004). In the current study, fecal corticosterone concentrations for the dominant female (SSL-03) were significantly different than those of the subordinate female (SSL-02). The subordinate female (SSL-02) does appear to elicit a stress response when confronted by the dominate female, as seen in the highest and third highest fecal corticosterone concentrations reported (Fig. 2-3b), although those peaks are considerably lower than the maximum levels of the dominant female. The highest peak in fecal corticosterone for SSL-03 corresponded with training sessions that included interactions with the subordinate female (Fig. 2-3c). Early studies, mainly with rats and mice, suggested that subordination was stressful (Bronson and Eleftheriou 1964; Louch and Higginbotham 1967; Bronson 1973). The territorial behavior of Weddell seals (*Leptonychotes weddelli*) during the breeding season showed that the dominant males had higher serum cortisol concentrations than subordinate males (Bartsh *et al.* 1992). Wild African wild dog (*Lycaon pictus*), dwarfed mongoose (*Helogale parvula*), and wolf (*Canis lupus*) studies also suggest that chronic stress is apparently a cost of social dominance rather than subordination (Creel and Creel 1996; Sands and Creel 2004). Typically, SSL-03 displaces SSL-02 by physically pushing her out of the way, or approaching the area of SSL-02, who then departs. SSL-03 has also been observed to steal food from SSL-02. Contact and interactions with the male were primarily through a fenced barrier. Fecal samples from any sea lions were not obtained when one or both of the females were housed in the same pool with the male due to the difficulty in identifying the sample.

It is important to identify if sex, social status, or season may influence, or instead are influenced by adrenal hormones or their metabolites. The small sample size currently only allowed the identification of a correlation of season and behavior in one male and two females. Only the male and one of the females displayed a correlation between exhibited behavior and corticosterone concentrations. Despite the small sample size, these results indicate that like other pinnipeds, Steller sea lions have a highly seasonal physiology that can be reflected in fecal corticosterone concentrations of both sexes. Fecal samples can be collected easily in the field all year round to monitor the adrenal health of Steller sea lions. Future studies to increase the sample size for each sex as well as to increase the age distribution will provide a critical baseline as a monitoring tool for samples collected in the wild.

LITERATURE CITED

Astherimer, L.B., W.A. Buttemer, and J.C. Wingfield. 1994. Gender and seasonal differences in the adrenocortical response to ACTH challenge in an Arctic passerine, *Zonotrichia leucophrys gambelii*. General and Comparative Endocrinology. 94: 33-43.

Boinski, S., S.P. Swing, T.S. Gross, and J.K. Davis. 1999. Environmental enrichment of brown capuchins (*Cebus paella*): Behavioral and plasma and fecal cortisol measures of effectiveness. American Journal of Primatology. 48: 49-68.

Bronson, F.H. and B.E. Eleftheriou. 1964. Chronic physiological effects of fighting in mice. General and Comparative Endocrinology. 4: 9-14.

Bronson, F.H. 1973. Establishment of social rank among grouped male mice: relative effects on circulating FSH, LH, and corticosterone. Physiology and Behavior. 10: 947-951.

Bushong, D.M., T.H. Friend, and D.A. Knabe. 2000. Salivary and plasma cortisol response to adrenocorticotropin administration in pigs. Laboratory Animals. 34: 171-181.

Creel, S. and N.M. Creel. 1996. Social stress and dominance. Nature. 379: 212.

Gardiner, K.J. and A.J. Hall. 1997. Diel and annual variation in plasma cortisol concentrations among wild and captive harbor seals (*Phoca vitulina*). Canadian Journal of Zoology. 75: 1773-1780.

Goldstein, D.S. 1995. Stress and science, in *Stress, Catecholamines and Cardiovascular Disease*, Goldstein, D.S. (ed.), Oxford University Press, Oxford, U.K., 3-55.

Hanson, J.D., M.E. Larson, and C.T. Snowdon. 1976. The effects of control over high intensity noise on plasma cortisol levels in Rhesus monkeys. Behavioral Biology. 16: 333-340.

Hunt, K.E., A.W. Trites, and S.K. Wasser. 2004. Validation of fecal glucocorticoid assay for Steller sea lions (*Eumetopias jubatus*). Physiology and Behavior. 80: 595-601.

Louch, C.D. and M. Higginbotham. 1967. The relation between social rank and plasma corticosterone levels in mice. General and Comparative Endocrinology. 8: 441-444.

Mashburn, K.L. and S. Atkinson. 2004. Evaluation of adrenal function in serum and feces of Steller sea lions (*Eumetopias jubatus*): influences of molt, gender, sample storage, and age on glucocorticoid metabolism. General and Comparative Endocrinology. 136(3): 371-381.

Monfort, S.L., K.L. Mashburn, B.A. Brewer, and S.R. Creel. 1996. Evaluating adrenal activity in African wild dogs (*Lycaon pictus*) by fecal corticosteroid analysis. Journal of Zoo and Wildlife Medicine. 29(2): 129-133.

Nelson, R.J. 2000. An Introduction to Behavioral Endocrinology. 2nd ed. Sinauer Associates, Inc. Sunderland, MA.

Oki, C. and S. Atkinson. 2004. Diurnal patterns of cortisol and thyroid hormones in the harbor seal (*Phoca vitulina*) during summer and winter seasons. General and Comparative Endocrinology. 136: 289-297.

Petrauskas, L., P. Tuomi, and S. Atkinson. 2004. Non-invasive monitoring of stress hormone levels of a female Steller sea lion (*Eumetopias jubatus*) pup undergoing rehabilitation. Journal of Zoo and Wildlife Medicine. *In Review.*

Place, N.J. and G.J. Kenagy. 2000. Seasonal changes in plasma testosterone and glucocorticosteroids in free-living male yellow-pine chipmunks and the response to capture and handling. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology. 170: 245-251.

Rea, L.D., M.A. Castellini, B.S. Fadely, and T.R. Loughlin. 1998. Health status of young Alaska Steller sea lion pups (*Eumetopias jubatus*) as indicated by blood chemistry and hematology. Comparative Biochemistry and Physiology Part A. 120: 617-623.

Romero, L.M, J.M. Reed, and J.C. Wingfield. 2000. Effects of weather on corticosterone responses in wild free-living passerine birds. General and Comparative Endocrinology. 118: 113-122.

St. Aubin, D.J., S.H. Ridgeway, R.S. Wells, and H. Rhinehart. 1996. Dolphin thyroid and adrenal hormones: circulating levels in wild and semidomesticated *Tursiops truncates*, and influence of sex, age, and season. Marine Mammal Science. 12(1): 1-13.

St. Aubin, D.J., and L.A. Dierauf. 2001. Stress and Marine Mammals, in *CRC Handbook of Marine Mammal Medicine*, Dierauf, L.A. and Gulland, F.M.D (ed.), CRC Press, Boca Raton, FL, 253-269.

Sands, J. and S. Creel. 2004. Social dominance, aggression and faecal glucocorticoid levels in a wild population of wolves, *Canis lupus*. Animal Behaviour. 67: 387-396.

Sapolsky, R.M. 1992. Neuroendocrinology of the stress-response, in *Behavioral Endocrinology*, Becker, J.B., S.B. Breedlove, D. Crews (ed.), MIT Press, Cambridge, MA, 287-324.

Sato, S., H. Kitamura, M. Mori, M. Fukazawa, M. Takeda, and K. Kadota. 1998. Adenocarcinoma of the lung in a Steller sea lion (*Eumetopias jubatus*). Journal of Veterinary Medical Science. 60(12): 1349-1351.

Strier, K.B., T.E. Ziegler, and D.J. Wittwer. 1999. Seasonal and social correlates of fecal testosterone and cortisol levels in wild male muriquis (*Brachyteles arachnoids*). Hormones and Behavior. 35: 125-134.

Teskey-Gerstl, A., E. Bamberg, T. Steineck, and R. Palme. 2000. Excretion of corticosteroids in urine and faeces of hares (*Lepus europaeus*). Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology. 170: 163-168.

Theodorou, J. and S. Atkinson. 1998. Monitoring total androgen concentrations in saliva from captive Hawaiian monk seals (*Monachus schauinslandi*). Marine Mammal Science. 14(2): 304-310.

Weiss, J.M. 1970. Somatic effects of predictable and unpredictable shock. Psychosomatic Medicine. 32(4): 397-408.

Wingfield, J.C., P. Devich, S. Sharbaugh, L.B. Astheimer, R. Holberton, R. Suydam, and K. Hunt. 1994. Seasonal changes of the adrenocortical responses in stress in redpolls, *Acanthis flammea*, in Alaska. The Journal of Experimental Zoology. 270: 372-380.

Wingfield, J.C. and M. Ramenofsky. 1997. Corticosterone and facultative dispersal in response to unpredictable events. Ardea. 85(1): 155-166.

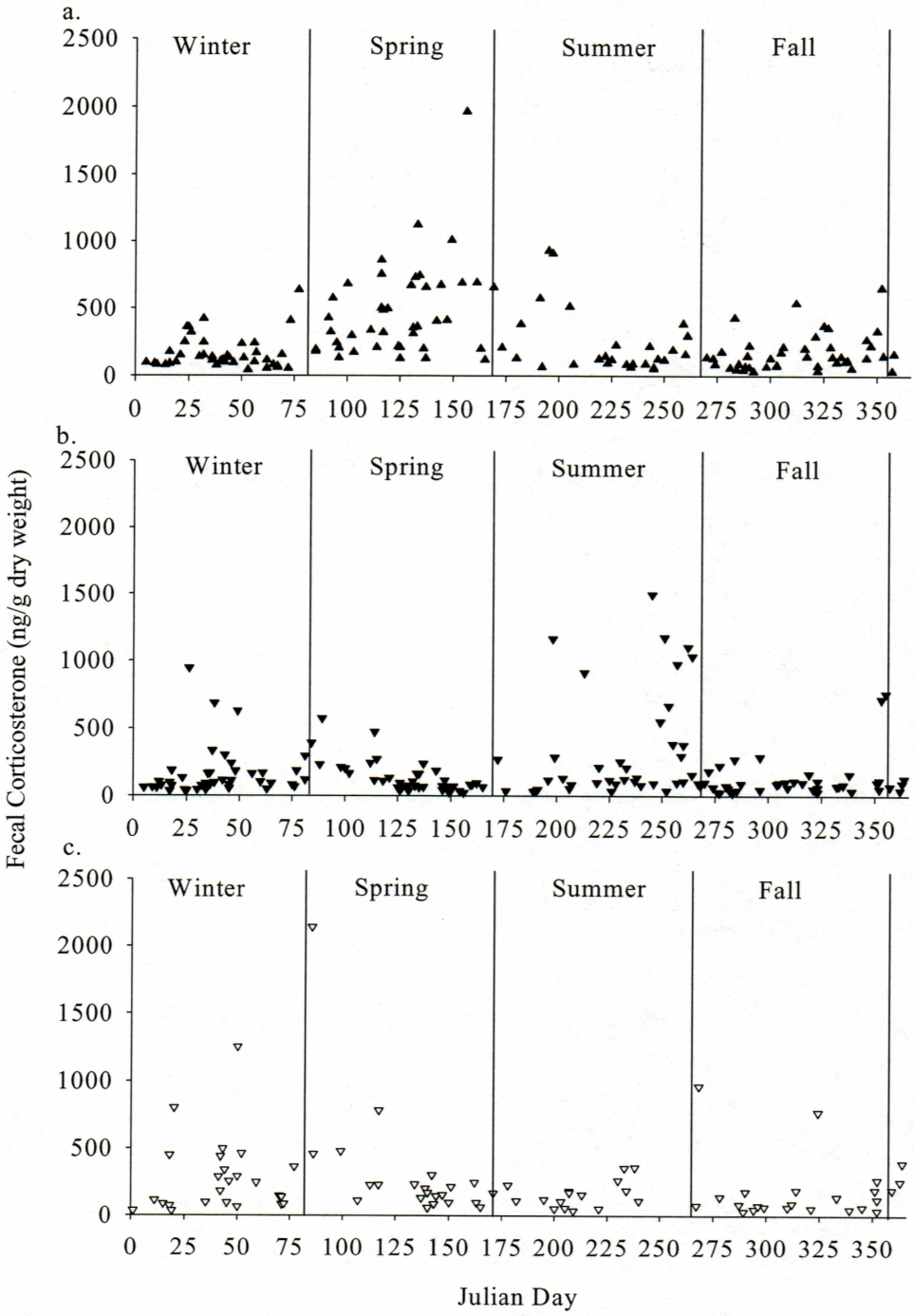

FIG. 2-1. Fecal corticosterone concentration (ng/g dry weight) for an adult male (a.) and two adult female (b. and c.) Steller sea lions during 2001-2004 based on Julian day. All years were combined and presented.

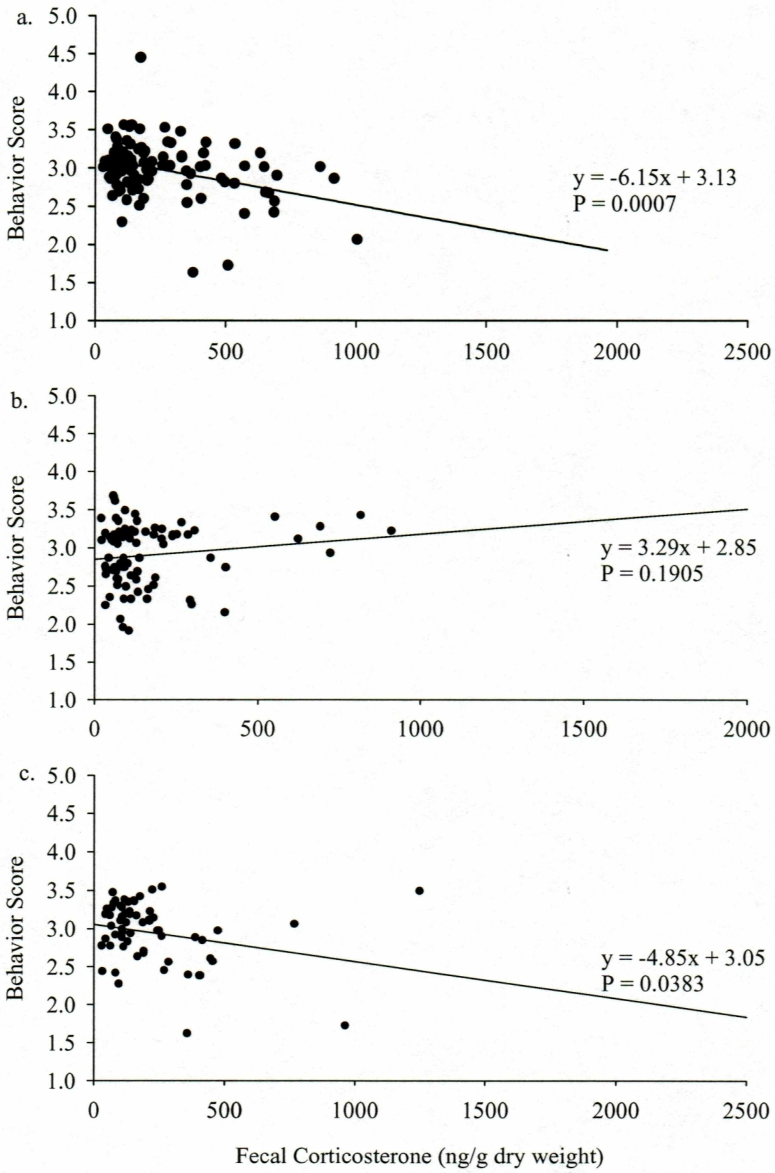

FIG. 2-2. Linear regression and analysis of variance for one adult male (a.) and two adult female (b., c.) Steller sea lions for mean weekly behavior score and fecal corticosterone concentration for samples collected 2001-2004

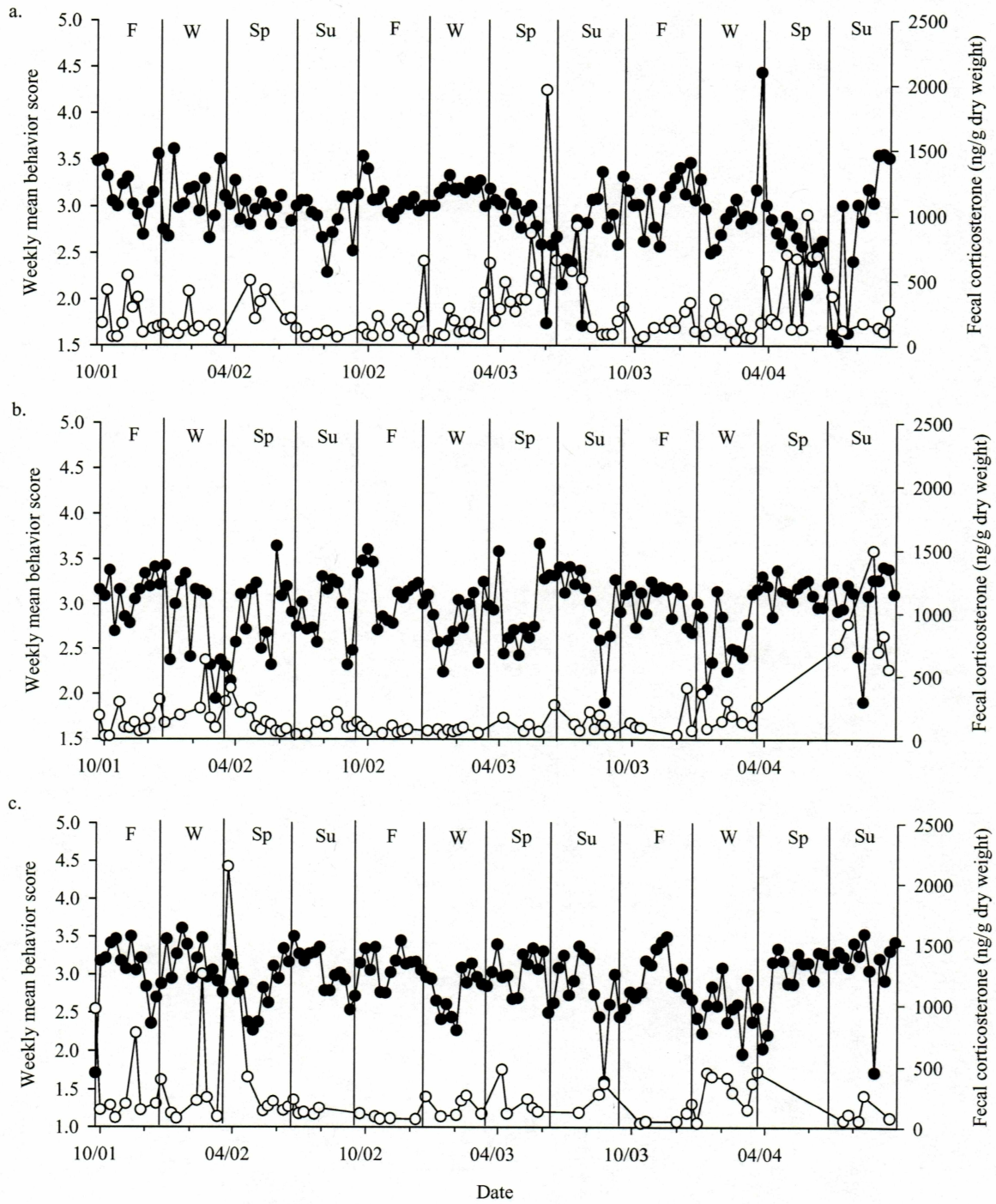

FIG. 2-3. Behavior score (-●-) and fecal corticosterone (-○-) concentrations (ng/g dry weight) for all three Steller sea lions: (a.) SSL-01, (b.) SSL-02, (c.) SSL-03.

Table 2-1. Fecal corticosterone concentrations and behavior score in Steller sea lions.

	Mean elevated behavior score (>500 ng/g)	Mean non-elevated behavior score (<500 ng/g)	T-Value	*P*
SSL-01 (m)	2.6 ± 0.48 n = 16	3.0 ± 0.33 n = 94	520.5	0.002
SSL-02 (f)	3.2 ± 0.17 n = 7	2.8 ± 0.41 n = 76	446.5	0.013
SSL-03 (f)	2.8 ± 0.96 n = 3	2.9 ± 0.37 n = 61	113.5	0.622

Chapter 3. Non-invasive monitoring of stress hormone levels of a female Steller sea lion (*Eumetopias jubatus*) pup undergoing rehabilitation [3]

ABSTRACT

Steller sea lions rarely strand in areas monitored by humans and there is little published data on the diseases, parasites, nutritional state, and stress levels of Steller sea lions (*Eumetopias jubatus*) in the wild. In May 2002, a female Steller sea lion pup (EJS-02-01) was sighted separated from her mother after strong storms in Southeast Alaska. After 5 days of observations, EJS-02-01 was transferred to the Alaska SeaLife Center (ASLC) in Seward, Alaska. During 11 months of rehabilitation at ASLC, body weights were monitored and opportunistic fecal samples (n=86) were analyzed for corticosterone concentrations. Fecal corticosterone concentrations ranged from 15-3805 ng/g for EJS-02-01. Peak corticosterone values reflected responses to acute stressors during rehabilitation. EJS-02-01 was successfully released at Gran Point, Alaska in April 2003. Fecal corticosterone assay monitoring provided a valuable tool to monitor various stressors and is useful in monitoring long-term situations, like rehabilitation.

Key Words: corticosterone, *Eumetopias jubatus*, non-invasive monitoring, rehabilitation, Steller sea lion.

[3] Petrauskas, L., P. Tuomi, S. Atkinson. 2005. Non-invasive monitoring of stress hormone levels of a female Steller sea lion (*Eumetopias jubatus*) pup undergoing rehabilitation. Journal of Zoo and Wildlife Medicine. In review.

BRIEF COMMUNICATION

Fecal corticosterone analysis is a non-invasive method to monitor glucocorticoids in organisms. Glucocorticoids are synthesized in the adrenal cortex, and targets include liver, lymphoid cells, thymus gland, and kidney.[5] Glucocorticoid release ensures the supply of energy to the individual via conversion of glycogen to energy rich glucose and the adaptive responses also include enhanced oxygen intake, memory and sensory function, with decreased blood flow to non-essential areas, inhibition of digestion, growth, immune function, reproduction, and pain perception.[4]

On 4 June 2002, a female Steller sea lion pup (EJS-02-01) was admitted to the ASLC rehabilitation program. Large storms on the Lowrie Island rookery in Southeast Alaska apparently washed the pup away from its mother. After five days of observations by the Alaska Department of Fish and Game, the pup was collected and transferred to Seward. Admit physical examination revealed that the pup was dehydrated, emaciated, and lethargic. For this study, fecal samples were collected opportunistically by the rehabilitation and veterinary staff. All samples were frozen at -20°C until extraction. To extract corticosterone from fecal material, all samples were fully mixed, aliquoted (~5 g), loaded onto a rotary evaporator (Speed-Vac Plus, SC110A; Savant Instruments, Holbrook New York), and dried without heat. Dried fecal samples were crushed into powder and 0.025 g (± .001) was weighed and extracted as previously described.[2,3] Methanol (MeOH) extractant (100 µl) was aliquoted into polypropylene tubes, dried under forced

air, reconstituted in 400 µl buffer for a final 1:4 dilution. Sample dilutions were stored frozen at -20°C until radioimmunoassay (RIA).

A double antibody RIA kit (MP Biomedicals (formerly ICN Biomedicals), Costa Mesa California) previously validated for use with Steller sea lion fecal extracts[2] was used for corticosterone analysis. Values from the RIA were corrected for dilution, extraction efficiency, weight of fecal material extracted, and expressed as ng/g dry weight. The RIAs were performed according to manufacturer's instructions with the exception that all volumes were halved and an additional standard was added to the curve (i.e., one-half the lowest standard) to increase sensitivity. Manufacturer cross-reactivity with other steroids was as follows: desoxycorticosterone (0.34%), testosterone (0.10%), cortisol (0.05%), aldosterone (0.02%) and less than 0.01% for all other steroids tested. Interassay coefficients of variation for two separate assay controls were 0.04 and 0.70% ($n = 2$ for all samples). Intra-assay coefficients of variation were <5% and assay sensitivity was 12.7 ng/tube.

Over the course of rehabilitation, the pup exhibited a nearly linear weight gain ($P < 0.0001$) with an admit weight of 16.4 kg and release weight of 104 kg (Fig. 1) and fecal corticosterone concentration ranged from 15-3805 ng/g (Fig. 2). These values were most likely reflective of a healthy individual responding to various acute stressors. Acute stress is defined as a controlled response to a stressor where the overall effect is not harmful.[1] Stress becomes detrimental, (i.e. chronic) when a state of distress causes the organism to respond to the stimuli in an excessive or uncontrolled manner.[7]

Fecal corticosterone concentrations during the summer months (May – August) for EJS-02-01 ranged from 15-3805 ng/g, and during the winter months (September – April) ranged from 82-2900 ng/g. The winter baseline for fecal corticosterone concentrations in adult male and female Steller sea lions was 88.5 ng/g, the maximum adrenal output was 1605.5 ng/g, and the elevated range in adrenal output was 500-1605.5 ng/g.[2] The summer baseline levels for adult female Steller sea lions was 29.8 ng/g and the maximum adrenal output was 1967 ng/g (Mashburn and Atkinson unpublished data).

Notable events during the 11 months of rehabilitation included admission to the facility, endoscopy and gastric lavage, intestinal infection with *Salmonella*, diet changes from milk-replacement (Milk Matrix 30/55 ®, Pet-Ag., Inc., Hampshire, Illinois 60140, USA) to fish mash to previously frozen fish (primarily herring) and then to live salmon, socialization with conspecifics, hot-branding and separation into pre-release quarantine. The largest peaks in fecal corticosterone occurred on 11 July 2002 and 8 April 2003 (Fig. 2), and corresponded to the *Salmonella* infection and hot branding, respectively.

The first fecal sample was collected 3d post transport to ASLC. The corticosterone concentration was above summer adult baseline (10.7-fold increase), but not above the 500 ng/g level considered to be elevated.[2] Fecal corticosterone measured in dairy cattle after transport also increased significantly.[6]

The initial recipe of milk-replacement formula may have contributed to an impaction that required medical intervention including anesthesia for endoscopy and gastric lavage. Fecal corticosterone rose to 830 ng/g 3d prior to the surgical procedure

and was 613 ng/g the day of surgery. Six and 10d post endoscopy, there were peaks of 521 and 793 ng/g, respectively, most likely reflecting prolonged recovery time (Fig. 2).

Over the next 7d, corticosterone concentrations of all but one sample fell below summer adult female baseline levels. The elevated sample corresponded to the first diagnosis of *Salmonella enteritis*. The largest peak analyzed was a 128-fold increase above adult baseline during treatment for the *Salmonella* infection. This may indicate that the adrenal function was ensuring adequate available energy and suppressing any inflammatory response to assist the body in fighting the infection. Antibiotic treatment was administered to treat the *Salmonella*, and the corticoids reversed and dropped below adult baseline through 9d after the infection had cleared.

For the next 2 mo., corticoid levels remained above summer adult female baseline levels (34 – 328 ng/g). Housing and bottle feeding of fish mash were routine and there were no unusual incidents reported in EJS-02-01's veterinary record and no observed impact on adrenal output. The first introduction of previously frozen herring along with fish mash did elicit a stress response of 937 ng/g and was considered elevated when compared with adult values. Removing fish mash completely from the diet did not elicit an adrenal response.

Two 9 yr. old female Steller sea lions housed permanently at the ASLC were introduced as conspecifics, beginning as short interactions with a fence barrier on 15 October 2002. Fecal corticosterone concentration 3d after the first introduction was 123 ng/g, which is very near winter adult baseline levels. A corticoid peak occurred 3d and 6d after the first introduction to the conspecifics without a barrier. From 15 November

2002 through 8 January 2003, the corticoid levels remained below 500 ng/g; 53% of those samples were below 250 ng/g. Over this time, either one or both of the adult females had contact on a daily basis with the pup. Overall, during this period of socialization, only 37.5% of the samples were considered elevated, indicating that socialization with conspecifics did not elicit a stress response except on a few occasions that were noted.

Overnight socializations began on 30 December 2002, and again on 3 January 2003. Both females were present when a small peak occurred in EJs-02-01's fecal corticosterone 5d after the second overnight session. A 9.6-fold increase occurred on 13 January 2003, 2d after the conspecifics were housed together with the pup in the pool for 24hrs. Additionally, the veterinary record noted a large amount of activity on the roof of the main building that seemed to have agitated both EJS-02-01 and the adult females during that period.

Housing changed on 1 April 2003, when EJS-02-01 was placed into quarantine as a pre-release procedure. A fecal corticoid sample peaked 24hr after the move to quarantine indicated a response to the change in housing. On 2 April 2003, EJS-02-01 was anesthetized and hot branded for post-release tracking purposes. A fecal sample was not collected until six days after the procedure. The fecal corticosterone concentration of that sample was the second highest recorded (33-fold increase above adult baseline). A sample was collected 10d after the hot branding and fecal corticosterone concentration returned to near adult baseline levels. EJS-02-01 was successfully released at Gran Point,

Alaska in April 2003, tracked via satellite telemetry and last sighted off of Gran Point on 3 September 2003.

The stress response is a series of complex reactions that can be measured non-invasively with fecal corticosterone. Fecal corticosterone assay monitoring provides a valuable addition to hematological diagnostics to ensure that a wild animal is responding to various stressors within normal ranges for that species and is useful in monitoring long-term situations, like rehabilitation. This non-invasive method to monitor stress longitudinally is especially well-suited for an endangered marine mammal, such as the Steller sea lion.

Acknowledgements: We thank the ASLC rehabilitation program and veterinary services staff, interns and volunteers; especially Millie Gray and Tim Lebling, and for sample collection and Kendall Mashburn for laboratory guidance. Support for this project was provided by the Alaska SeaLife Center Steller Sea Lion Program with funding through the National Marine Fisheries Service.

LITERATURE CITED

1. Goldstein, D.S. 1995. Stress and science, in *Stress, Catecholamines and Cardiovascular Disease*, Goldstein, D.S. (ed.), Oxford University Press, Oxford, U.K., 3-55.

2. Mashburn, K.L., and S. Atkinson. 2004. Evaluation of adrenal function in serum and feces of Steller sea lions (Eumetopias jubatus): influences of molt, gender, sample storage, and age on glucocorticoid metabolism. General and Comparative Endocrinology. 136(3): 371 - 381.

3. Monfort S.L., K.L. Mashburn, B.A. Brewer, and S.R. Creel. 1996. Evaluating adrenal activity in African wild dogs (Lycaon pictus) by fecal corticosteroid analysis. Journal of Zoo and Wildlife Medicine. 29(2): 129 - 133.

4. Nelson, R.J. 2000. An Introduction to Behavioral Endocrinology. 2nd ed. Sinauer Associates, Inc. Sunderland, MA.

5. Norman, A.W., and G. Litwack. 1987. Hormones. Academic Press, Inc. San Diego, CA.

6. Palme, R., C. Robia, W. Baumgartner, and E. Möstl. 2000. Transport stress in cattle as reflected by an increase in faecal cortisol metabolite concentrations. Veterinary Record. 146: 108 - 109.

7. St. Aubin, D. J., and L.A. Dierauf. 2001. Stress and Marine Mammals, in *CRC Handbook of Marine Mammal Medicine*, Dierauf, L.A. and Gulland, F.M.D (ed.), CRC Press, Boca Raton, FL, 253-269.

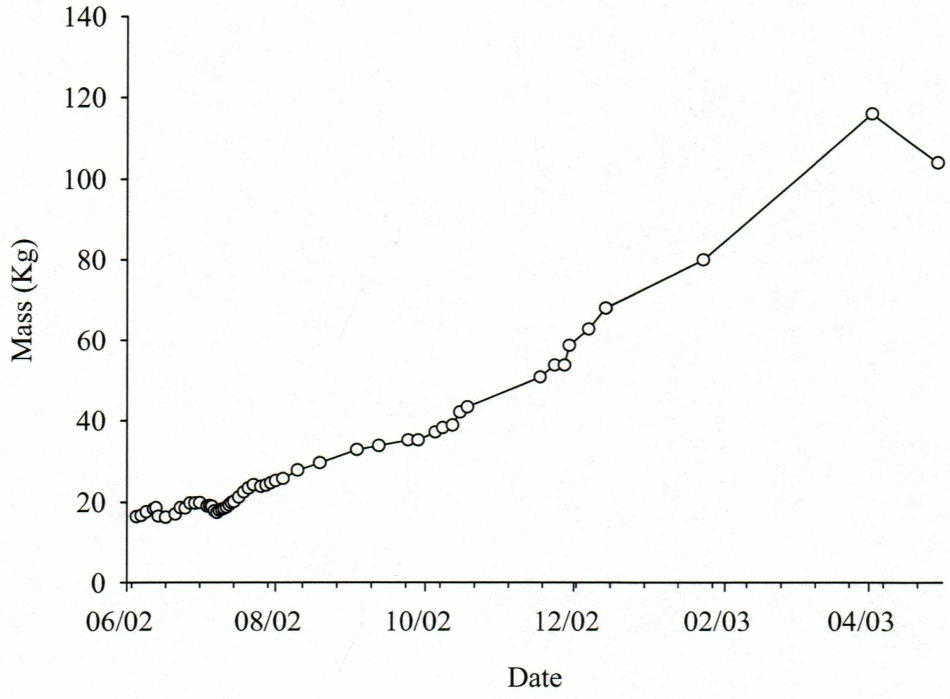

FIG. 3-1. Mass of EJS-02-01 during 11 months of rehabilitation at ASLC.

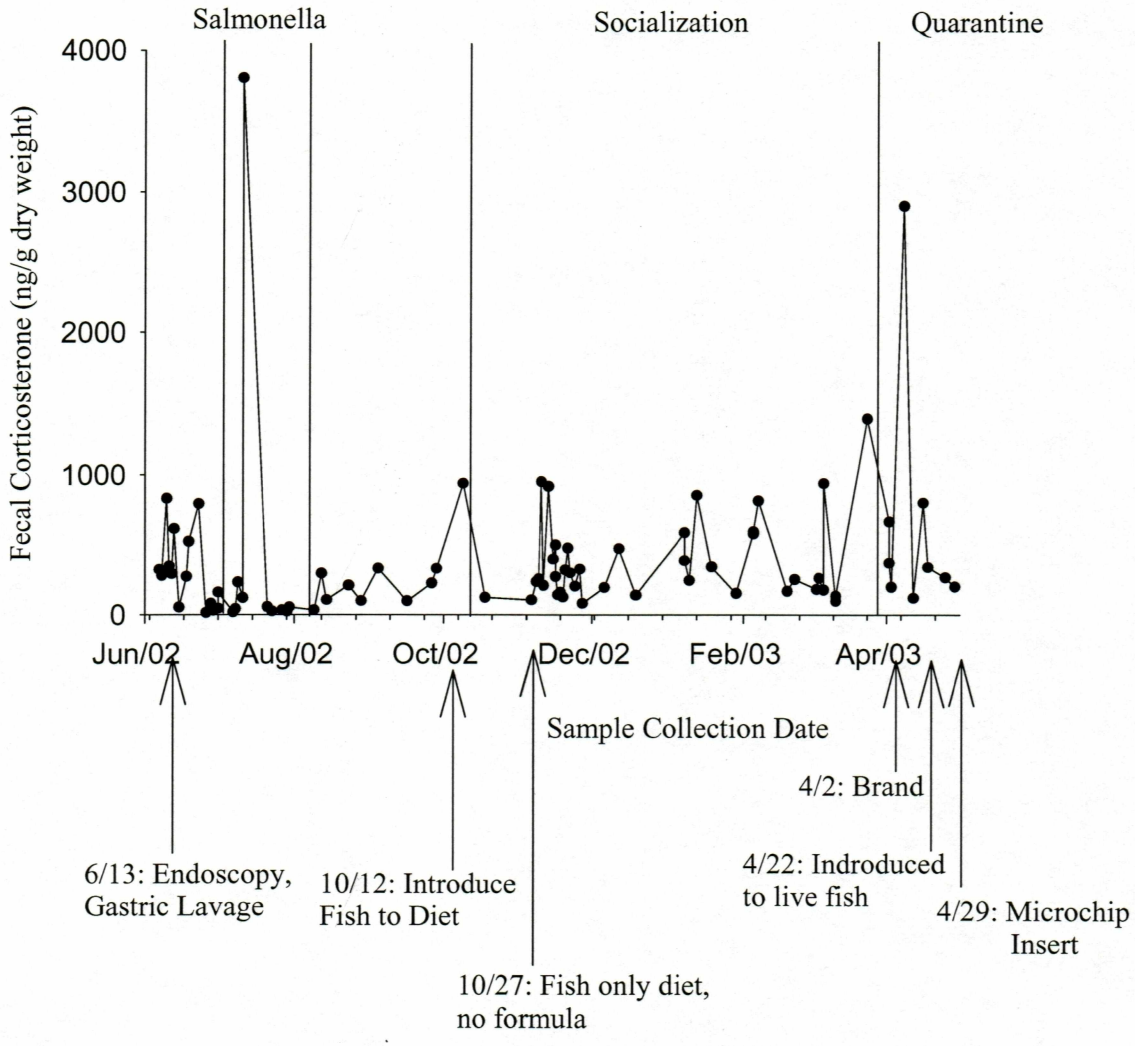

FIG. 3-2. Fecal corticosterone concentrations for a young Steller sea lion, EJS-02-01, during 11 months of rehabilitation at ASLC.

General Conclusions

The commercially available radioimmunoassay kits for cortisol and corticosterone (Diagnostic Products and MP Biomedicals, respectively) were biologically and functionally valid for use in monitoring those glucocorticoids in sera and fecal samples from California sea lions. Fecal corticosterone is a medium that is widely used for monitoring stress non-invasively and longitudinally in mammals, and appears to be equally as useful in monitoring stress, or well-being of otariids.

Both compromised and non-compromised California sea lions that underwent rehabilitation at TMMC displayed an acute stress response to the specific procedures that each individual underwent. Those procedures included physical restraint for a blood draw, isofluorane anesthesia only, minor invasive surgical procedures and major invasive surgical procedures. Overall, 23 of 34 sea lions displayed an increased adrenal response to the particular procedure that they underwent. Of those 23 animals, 14 were healthy at the time of the procedure and only four animals were not released successfully. The successful response to rehabilitation procedures and eventual release of most of these animals indicated that those procedures were not chronically stressful and the animals' response was a normal acute response to something "unnatural" occurring. By collecting fecal samples, the results were strengthened due to those collections being non-invasive and, therefore, not evoking a stress response.

Similarly, fecal corticosterone concentrations for a female Steller sea lion pup undergoing rehabilitation were reflective of a healthy individual responding to various acute stressors. Those acute stressors included: admission to the facility, endoscopy and

gastric lavage, a *Salmonella* intestinal infection, diet and housing changes, socialization with adult conspecifics, hot-branding and separation into pre-release quarantine. Using fecal corticosterone monitoring in this case provided useful non-invasive monitoring to a long-term rehabilitation situation. This method monitored the overall well-being and the response to acute stressors especially well for a threatened species.

Captive, healthy and reproductively intact Steller sea lions can provide a baseline for free-ranging studies due to the bountiful amount of known fecal samples that can be collected. These samples can be correlated to the age of the animal, season, sex and behavior of the individual. Only the male and one of the females displayed a correlation between exhibited behavior and corticosterone concentrations. The results also indicated that Steller sea lions have a highly seasonal physiology that can be measured in fecal corticosterone concentrations for both males and females.

California and Steller sea lions both produce corticosterone from the adrenal cortex in response to a perceived stressor. Corticosterone for both of these species stays in its original state through fecal deposition, as demonstrated via HPLC in this study. The use of surrogate species in developing and testing new instrumentation or in validating analytical procedures is commonly practiced. The similar anatomical and physiological characteristics, as well as, the overlap in habitat of these two species of sea lions indicate that the non-threatened/endangered California sea lion would be a likely choice for surrogacy to the Steller sea lion.